

Review Article

Indian J Med Res 127, March 2008, pp 274-286

Role of calcium-sensing receptor in bone biology

Kunal Sharan, J.A. Siddiqui, Gaurav Swarnkar & N. Chattopadhyay

Division of Endocrinology, Central Drug Research Institute, Lucknow, India

Received October 5, 2007

Bone turnover helps accomplish long-term correction of the extracellular calcium (Ca^{2+}_o) homeostasis by the actions of osteoblasts and osteoclasts. These processes are highly regulated by the actions of hormones, most prominently parathyroid hormone (PTH), the release of which is a function of the Ca^{2+}_o , and is regulated by the action of the Ca^{2+} -sensing receptor (CaR) in the parathyroid gland. Various mutations of the *CaR* gene give rise to gain or loss of functions leading respectively to hypo- or hypercalcaemic conditions. CaR could conceivably be a target for local changes in the Ca^{2+}_o in the bone microenvironment thereby acting as a 'growth factor' in various cells residing in the bone marrow. This review discusses about the roles of the CaR in bone. In osteoblasts, CaR promotes its proliferation, differentiation and mineralization. In osteoclasts, CaR mediates high Ca^{2+}_o -stimulated osteoclast differentiation as well as osteoclast apoptosis. CaR regulates localization of haematopoietic stem cells from the foetal liver to endosteal niche, the so-called homing. Although the CaR plays a key role in the defense against hypercalcaemia, its function can be aberrant in humoral hypercalcaemia of malignancy in which CaR activation stimulates secretion of parathyroid hormone-related peptide (PTHrP) secretion. Increased levels of PTHrP cause a vicious hypercalcaemic state resulting from its increased bone-resorptive and positive renal calcium reabsorbing effects give rise to hypercalcaemia. CaR mediates a variety of functions of Ca^{2+}_o in the bone microenvironment under both normal and pathological conditions.

Key words Bone microenvironment - calcium sensing receptor – *CaR* gene mutation - hypercalcaemia - osteoblasts

Introduction

Intracellular calcium (Ca^{2+}_i) is a key intracellular second messenger that plays a pivotal role in controlling various cellular processes such as secretion, differentiation, proliferation, motility, and cell death. In contrast, extracellular (Ca^{2+}_o) is crucial for a spectrum of physiological phenomena, including blood coagulation, neurotransmitter release, muscle function and maintenance of skeletal integrity.

Upon the activation of excitable cells, they undergo rapid changes in Ca^{2+}_i concentration, whereas Ca^{2+}_o is maintained within a narrow physiological range (1.1–1.3 mM). Circulating levels of Ca^{2+} are controlled mostly by the calcitropic hormones notably, parathyroid hormone (PTH) and vitamin D. PTH is secreted from parathyroid gland (PTG) cells and acts on the bone, the kidney, and indirectly through vitamin D, the small intestinal cells to mobilize Ca^{2+}_o . The secretion of PTH is rapidly decreased by increases in

Ca^{2+}_o because of a reciprocal relationship between circulating PTH and Ca^{2+} levels¹.

The calcium sensing receptor (CaR) is a key mediator of direct actions of Ca^{2+} on parathyroid and kidney and regulates homeostatic responses that restore Ca^{2+} to its normal level²⁻⁴. Parathyroid chief cells are the most renowned calcium sensing cells in the body, as reflected by the crucial inverse sigmoidal relationship between PTH and Ca^{2+}_o ⁵. The issue of Ca^{2+} sensing receptor began when Nemeth and colleagues observed that Ca^{2+} , other di- and trivalent cations, and organic polycations that poorly enter inside the cell, rapidly increased Ca^{2+}_i of cultured bovine PTG cells, suggesting the existence of a membrane-associated cation-sensing mechanism, possibly a receptor, that enables these cells to detect and respond to small changes in Ca^{2+}_o ⁶⁻⁸. Our understanding of the emerging role of Ca^{2+}_o not only as a physiologically indispensable ion but also as a signaling entity (extracellular first messenger) has advanced remarkably following the cloning of the CaR from bovine parathyroid gland in 1993². Since the identification of this clone (named BoPCaR for bovine parathyroid calcium-sensing receptor), the receptor for Ca^{2+}_o has been identified in numerous species including salamander⁹, rat¹⁰, human¹¹, and in a wide range of tissues including bone, intestine, pancreas and brain³.

The CaR has the “signature” G protein-coupled receptor (GPCR) topology, a seven-membrane-spanning, “serpentine” domain, as well as a large extracellular ligand-binding domain (ECD) and an intracellular COOH-terminal domain. The large ECD is topologically related to the Venus flytrap structure of bacterial periplasmic binding proteins and exhibits several clusters of acidic amino-acid residues, which probably interact with Ca^{2+} in the millimolar range through electrostatic interactions. The functional CaR on the cell surface forms a homodimer through covalent intermolecular disulphide bonds between cysteine residues in the ECD and is considered to contribute to the high degree of receptor cooperativity^{12,13}.

The events that occur downstream of CaR activation are complex and can be mediated via several different signaling pathways. In parathyroid cells, the signaling of the CaR involves coupling through G_i proteins (to adenylate cyclase) and G_q/G_{11} proteins (to phospholipase C)¹⁴.

Diseases caused by calcium sensing receptor gene mutation

Cloning of the CaR was rapidly followed by the identification of inherited Ca^{2+}_o -sensing disorders

resulting from *CaR* gene mutations. Inactivating and activating mutations of the *CaR* gene give rise to hypercalcaemic and hypocalcaemic disorders, respectively¹⁵⁻¹⁷.

Disorders with generalized resistance to extracellular calcium

Disorders with generalized resistance to Ca^{2+}_o occur due to inactivating mutations of the *CaR*. The two inherited disorders of hypercalcaemia - familial hypocalciuric hypercalcaemia (FHH) and neonatal severe hypercalcaemia (NSHPT) identified immediately after the cloning of CaR were found to be due to inactivating mutation of the CaR gene^{18,19}.

Familial hypocalciuric hypercalcemia (FHH): FHH is inherited as an autosomal-dominant trait with a high penetrance of over 90 per cent^{20,21}. The gene was first mapped to the long arm of the chromosome 3 (band q21-24). This locus harbours the disease gene in >90 per cent cases reported to date²².

FHH is a rare disorder of mineral metabolism characterized by lifelong, mild to moderate but usually asymptomatic hypercalcaemia. Another feature of this disease is the presence of inappropriately low rates of urinary calcium excretion (a calcium to creatinine clearance ratio of <0.01), mildly elevated level of magnesium and a nonsuppressed circulating levels of PTH, phosphorus and vitamin D levels regardless of the presence of hypercalcaemia^{20,21}. The disorder is considered to be benign as patients with FHH are usually asymptomatic. However, there is an increased prevalence of chondrocalcinosis with advancing age and occasional cases of acute pancreatitis have been reported^{20,23}.

Individuals with FHH display abnormal parathyroid and renal responsiveness to Ca^{2+}_o . Induction of hyper- and hypocalcaemia in these subjects reveals a right-shifted set-point for calcium-regulated PTH secretion²⁴. In other words, the level of serum calcium required to suppress the serum PTH level by half is higher than that required in normal subjects. Thus for any given serum level of calcium, FHH patients have a higher PTH concentration than normal individuals.

Since the initial description of mutations in the *CaR* as the cause of most cases of FHH in 1993, more than 100 different mutations have been identified so far, with each family generally having its own unique mutation (see <http://www.CaRdb.mcgill.ca/>). These mutations comprise missense, nonsense, deletion, insertion, deletion/insertion and splice site mutations.

Neonatal severe hyperparathyroidism: Due to severe primary hyperparathyroidism with enlargement of all four parathyroid glands, the degree of hypercalcaemia in NSHPT is usually more severe than that observed in FHH, and this disorder can be fatal if parathyroidectomy is not carried out within the first weeks of life. Bone demineralization, often accompanied by multiple fractures of long bones and ribs may be present. Most cases present with failure to thrive, anorexia, constipation, and, in some infants, respiratory distress related to chest deformity arising from fractures of several ribs. Skeletal radiology shows evidence of severe hyperparathyroidism, often with multiple fractures²⁵. Serum calcium levels typically range between 14–20 mg/dl, although values as high as 30.8 mg/dl have been reported²⁵. PTH levels are usually (but not always) very high, and the parathyroid glands are enlarged, often markedly so, exhibiting chief cell hyperplasia on histology^{26,27}.

Some infants with NSHPT represent the homozygous form of FHH²⁸, or, as seen in one case, a compound heterozygote in which a different inactivating *CaR* mutation was inherited from each parent²⁹. In some cases, NSHPT may be caused by the presence of heterozygous inactivating mutations of the *CaR*, either in a familial setting or as a *de novo* mutation in the offspring of normal parents, and possibly in these cases the mutant *CaR* can exert a dominant negative action, impairing the function of the normal receptor³⁰.

Disorders with generalized oversensitivity to extracellular calcium

Autosomal dominant hypocalcaemia (ADH): A heterozygous germline activating missense mutation of the human *CaR* inhibits PTH secretion and reduces renal calcium reabsorption at an inappropriately low serum calcium concentration, leading to hypocalcaemia, relative hypercalciuria and an inappropriately low serum PTH that characterizes subjects with ADH or sporadic hypocalcaemia³¹. The rare genetic hypocalcemic disorder, ADH, is caused by activating mutation ('gain-of-function') of *CaR*¹⁷. When the hypocalcaemia is treated by administering calcium and/or vitamin D sterols, marked hypercalciuria due to increased sensitivity of renal *CaR* often results in nephrolithiasis or nephrocalcinosis. In the 13 kindreds reported with this syndrome of ADH, ten different mutations of the *CaR* have been found in 11 families^{28,32,33}. All of these ten mutations were missense, and eight of them were located within the extracellular

domain of the receptor and only one was found in a transmembrane domain, with the remaining mutation being located in the first extracellular loop^{17,30,32,33}.

“Knockout” mouse model for *CaR* gene phenotype

Another evidence highlighting indispensable role of the *CaR* was obtained with the availability of mice with targeted disruption of *CaR* gene³⁴. The mouse model which was heterozygous and homozygous for *CaR* “knockout” exhibited a clinical and biochemical features very much similar to FHH and NSHPT, respectively. About 50 per cent reduction of *CaR* was exhibited in parathyroid and kidney of heterozygous mice and was phenotypically unremarkable, exhibiting normal fertility and life span. Their serum calcium averaged 10.4 mg/dl: 10 per cent higher than in normal littermates. They also exhibited mild (~10-15%) but significant increases in Mg^{2+} , approximately 50 per cent elevations in serum PTH, and urinary Ca^{2+} levels slightly lower than in normal mice, despite their hypercalcaemia. Thus mice heterozygous for *CaR* knockout exhibit many of the phenotypic and biochemical features of FHH.

In contrast to this, mice homozygous for *CaR* “knockout” were severely hypercalcaemic, averaging 14.8 mg/dl. Their Mg^{2+} was slightly higher than in heterozygotes, and serum PTH was about 10-fold higher than in normal mice: an increase comparable to that in NSHPT. Despite their severe hypercalcaemia, urinary Ca^{2+} in homozygous mice was lower than in normal mice. The homozygous mice, as in NSHPT, also exhibited marked parathyroid hyperplasia, supporting the *CaR*'s role in suppressing parathyroid proliferation. Skeletal x-rays showed reductions in density, kyphoscoliosis and bowing of long bones. Thus mice homozygous for *CaR* knockout provide an animal model of NSHPT³⁴.

Pharmacological modulators of *CaR*

Just a decade after the *CaR* was identified, pharmacological manipulation of the *CaR* has entered the clinic. For hyperparathyroid states, calcimimetics, which increase activation of the *CaR*, have been licensed in Europe and the USA. Calcilytics, which decrease *CaR* function and increase secretion of parathyroid hormone (PTH), might allow the anabolic effects of PTH on bone to be harnessed for the prevention and treatment of osteoporosis (Fig. 1).

Calcimimetic agents: Calcium is not a unique ligand for the *CaR*, although it is the only one with a

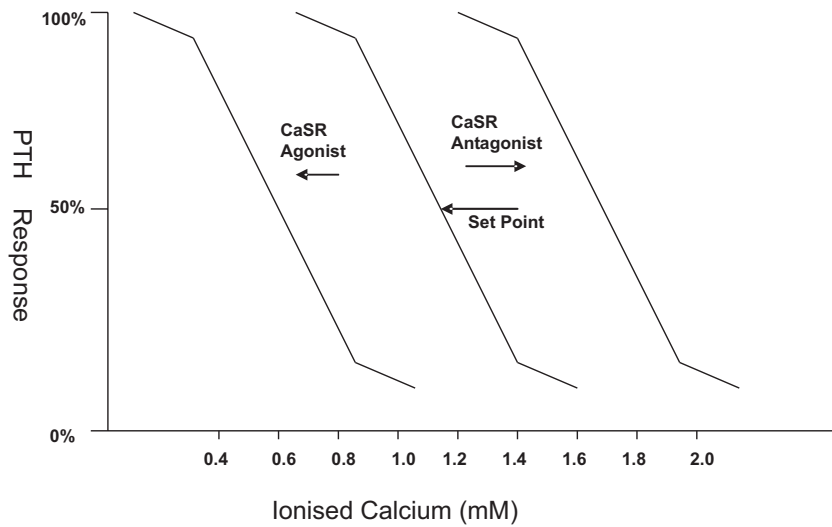


Fig. 1. Effect of pharmacological modulators of the CaR. Calcimimetic given to a normal (normocalcaemic) person will sensitize the CaR and shift the set point for Ca^{2+} mediated PTH suppression to the left. This means that less Ca^{2+} is required to suppress PTH and the person will be hypocalcaemic, like in the case of ADH. On the other hand, the same person given calcilytic will have his/her set point for Ca^{2+} mediated PTH suppression shifted to right resulting in hypercalcaemia, like in the case of FHH.

convincing physiological role. Type I calcimimetics directly activate the CaR, and include calcium and other divalent and trivalent cations, spermine, aminoglycoside antibiotics, and some polyvalent amino acids and peptides. Type II agents are not strictly agonists but positive allosteric modulators, increasing the receptor's sensitivity to ambient Ca^{2+} (or other type I agents) through binding within the transmembrane region³⁵. The development of type II compounds proceeded with an eye on future use in hyperparathyroid states. The prototype drug was NPS R-568. Preclinical and clinical studies showed efficacy in primary and secondary hyperparathyroidism, and parathyroid carcinoma, before unpredictable pharmacokinetic property led to its withdrawal in favour of cinacalcet³⁶.

Calcilytics: Calcilytics decrease the sensitivity of the CaR to calcium, thereby increasing PTH secretion³⁷, and will probably prove to be more than just pharmacological tools. PTH has powerful effects on bone remodelling. Sustained elevations of PTH, as in hyperparathyroid states, have a net catabolic effect on bone, favouring resorption. Short bursts of PTH are anabolic, favouring bone formation. This discrepancy has been exploited therapeutically, with intermittent bolus doses of synthetic PTH increasing bone mass and decreasing fracture rates in osteoporosis³⁸. Therefore, it is plausible that intermittent administration of a calcilytic could mirror this cyclical pattern in endogenous PTH, promoting anabolic over catabolic actions.

CaR and bone remodelling

Bone is the major sink and store for calcium and it fulfils essential roles in the maintenance of Ca^{2+}_o within its homeostatic range (1.1-1.3 mM). In condition of acute hypercalcaemia or hypocalcaemia, Ca^{2+} is rapidly transported into or out of bone. Ca^{2+} released from the bone acts as long-term correction of the Ca^{2+}_o by the metabolic actions of osteoblast and osteoclast, which incorporate or release Ca^{2+} from bone respectively³⁹. *In vitro* studies indicate that bone cells also directly respond to increasing and decreasing Ca^{2+}_o in their vicinity, independently of the systemic factors. But the molecular mechanisms which enable the bone cells to sense and respond to Ca^{2+}_o are not clear. Like the parathyroid cells, bone cells also express the CaR, and accumulating evidence indicates the involvement of this receptor in their responses to the changing extracellular ionic environment³⁹.

The bone remodelling cycle involves a complex series of sequential steps that are highly regulated. The "activation" phase of remodelling is dependent on the effects of local and systemic factors on mesenchymal cells of the osteoblast lineage. These cells interact with haematopoietic precursors to form osteoclasts in the "resorption" phase. Subsequently, there is a "reversal" phase during which mononuclear cells are present on the bone surface. They may complete the resorption process and produce the signals that initiate formation. Finally, successive waves of mesenchymal cells

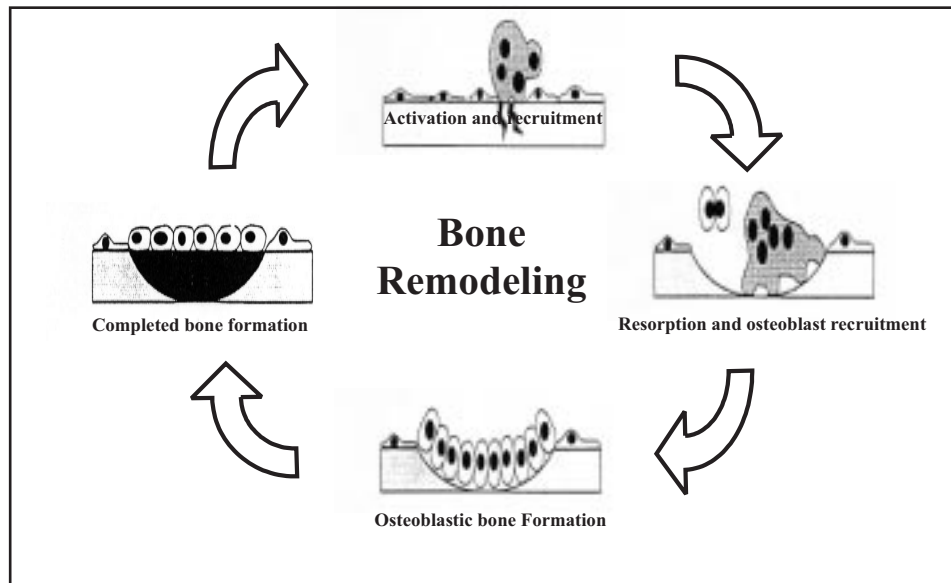


Fig. 2. Bone remodelling cycle. Preosteoclasts are stimulated and differentiated under the influence of various cytokines and growth factors to become mature and active osteoclasts, which in turn are recruited to bone resorption site. Osteoclasts resorb bone mineral and matrix. Osteoblast precursor cells in response to various systemic and local stimuli proliferate and differentiate into mature osteoblasts, migrate into the resorption lacuna and deposit matrix proteins and mineral.

differentiate into functional osteoblast, which lay down matrix in the “formation” phase (Fig. 2).

Increase in Ca^{2+}_o concentration has been shown to inhibit osteoclastic bone resorption and stimulate proliferation and chemotaxis of osteoblasts. Indeed, *in vitro* studies has shown that high calcium induces chemotaxis of human peripheral blood monocytes⁴⁰ (which differentiate into osteoclast⁴¹) and chemotaxis as well as DNA synthesis of mouse osteoblastic MC3T-E1 cells^{40,42-44} and in differentiated osteoblasts⁴⁵. Therefore, calcium released by bone resorption may have important roles in the coupling of bone resorption and bone formation (Fig. 3). Although both osteoclasts and osteoblasts have calcium-sensing mechanisms, the responsible molecule in these cells seems to be different. Functional and histological studies show that calcium-sensing mechanism in osteoclasts is a ryanodine receptor-like molecule in plasma membrane. In contrast, calcium-sensing mechanism in osteoblast has similar functional property to parathyroid CaR, but there is a report suggesting a different molecule mediating the function of high Ca^{2+} in osteoblasts⁴⁶. In addition, several bone marrow cells such as osteoblast^{43,47-49}, monocytes-macrophages^{50,51}, osteoclast⁵² express CaR, which suggest that the receptor is involved in physiological responses to local high Ca^{2+}_o concentration in the skeletal microenvironment.

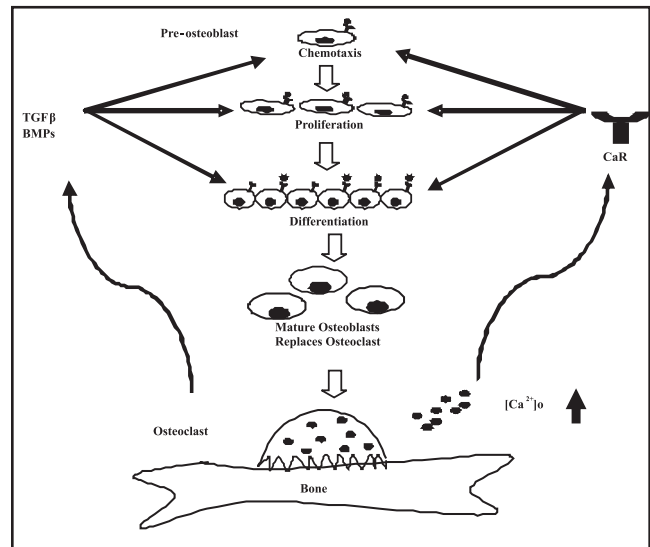


Fig. 3. Osteoblast activation following osteoclast function. Bone matrix is a rich source of various growth factors and cytokines. In addition, body's 99 per cent calcium is stored in bone. Osteoclastic activity results in the release of various growth factors such as TGF β and BMPs. These factors have several osteoblast stimulatory effects. However, bone resorption will also give rise to release of Ca^{2+} that could act as a local growth factor by acting via the CaR in the osteoblasts. Therefore, elevated levels of Ca^{2+}_o by the action of osteoclast on bone along with other factors will induce chemotaxis followed by proliferation and differentiation of osteoblast through CaR activation which finally after maturation displaces osteoclasts at bone resorption site thus maintaining calcium homeostasis.

CaR and osteoblast

Whether the cloned CaR is present in osteoblasts has been a source of considerable controversy. Earlier studies failed to show its expression in MC3T3-E1⁵³ and SAOS-2 osteoblastic cells at gene and protein level. On that basis, it was suggested that high calcium could act on osteoblast by a calcium sensing mechanism other than that of parathyroid CaR⁴². Later on more refined and sensitive techniques collectively proved that the CaR is expressed in several osteoblastic cells such as in MC3T3-E1⁴³, SAOS-2, UMR-106⁵⁴ and MG-63⁵⁵. These cell lines show characteristics of osteoblast. MG-63 with high ALP but low osteocalcin activity on treatment with 1, 25 (OH)₂ vitamin D₃ resemble undifferentiated osteoblast precursor. In addition, Chang *et al*⁴⁷ have shown CaR at mRNA and protein levels in osteoblast in mouse, rat and bovine bone. Exposure of MC3T3-E1 cells to high Ca²⁺_o (up to 4.8 mM) or the polycationic CaR agonists, neomycin and gadolinium (Gd³⁺), stimulated both chemotaxis and DNA synthesis in MC3T3-E1 cells, suggesting CaR in these osteoblasts could play a key role in regulating bone turnover by stimulating the proliferation and migration of such cells to sites of bone resorption as a result of local release of Ca²⁺_o⁴³.

However, the ability of the osteoblasts obtained from CaR-*null* mice to sense cations and amino acids suggested that a different molecule other than the parathyroid CaR could be involved in osteoblast calcium sensing⁵⁶. Recently, another family C GPCR, GPCRC6A, was found to sense Ca²⁺_o, albeit at levels well above those present in various extracellular fluids⁵⁷. Alignment of GPCRC6A with CaR revealed conservation of both calcium and calcimimetic binding sites. Reverse transcription-PCR analyses showed that mouse GPCRC6A is widely expressed in mouse tissues; including bone, calvaria, and the osteoblastic cell line MC3T3-E1⁵⁷. Osteocalcin, a calcium-binding protein that is highly expressed in bone, dose-dependently stimulated GPCRC6A activity in the presence of calcium but inhibited the calcium-dependent activation of CaR. These results suggest that GPCRC6A could be an osteoblast specific calcium sensor however, it is currently conjectural whether this receptor qualifies as a *bona fide* CaR⁵⁷.

Recently, two studies showed expression of CaR in rat calvarial osteoblasts and in human osteoblasts^{58,59}. These studies used molecular and pharmacological tools to show that the effect of Ca²⁺_o is mediated by the parathyroid CaR. CaR has been shown to modulate proliferation, differentiation and mineralization of

osteoblasts. High Ca²⁺_o has been shown to stimulate cyclin D genes and early oncogenes, *c-fos* and *egr-1* in osteoblasts that could mediate the mitogenic effect of the CaR in these cells. Strontium ranelate is a recently developed drug for postmenopausal women with osteoporosis. In osteoblasts, Sr²⁺ has recently been shown to act via the CaR and stimulates osteoblast proliferation⁶⁰.

Regarding the signaling mechanism in osteoblasts, following the CaR activation, involvement of PLC pathway as observed in parathyroid and kidney was found to be equivocal. Whereas one report showed activation of PLC³, other report failed to show such effect⁶¹. In MC3T3-E1 cells, elevated Ca²⁺_o and other type I calcimimetics resulted in the activation of p42/44 MAPK and p38 MAP kinase pathways⁶². In rat primary osteoblasts, CaR-stimulated proliferation is mediated via a JNK pathway⁵⁸. Recently it has been found that CaR mediates the opening of Ca⁺⁺-activated K⁺ channel in MC3T3-E1 cells⁶³ thereby suggesting that the CaR could be involved in the maintenance of the cellular ionic milieu and electrical polarization of osteoblasts.

Role of calcium sensing receptor in osteoclast

Osteoclasts are large, multinucleated, highly specialized bone-resorbing cells derived from the haematopoietic monocyte-macrophage lineage. Two key cytokines, receptor activator of (NF-κB) (RANK) ligand (RANKL) and macrophage colony stimulating factor (M-CSF) regulate osteoclastogenesis. RANKL, a member of the Tumour Necrosis Factor (TNF) family, is produced by osteoblasts, stromal cells, and B and T cells⁶⁴⁻⁶⁸. RANKL stimulates osteoclast precursors to differentiate via binding to the receptor, RANK⁶⁹. Since monocytes-macrophages are known to have the capacity to fuse with one another and to differentiate into mature functional osteoclast under specific culture condition, the existence of the CaR expression in monocytes-macrophages raises the possibility that CaR expression could persist throughout the differentiation of monocytes-macrophages to mature osteoclast. This receptor may also have roles in the function of the cells at various differentiation stages.

Importance of calcium in the osteoclast function has been revealed by several reports⁷⁰⁻⁷⁵. Exposing the osteoclasts to millimolar levels of Ca²⁺ results in dramatic cell retraction followed by a profound inhibition of bone resorption⁷⁶⁻⁷⁸. Osteoclasts first attach to mineralized surfaces and actively resorb bone, releasing calcium into the extracellular environment⁷⁹. Resultant levels of

elevated Ca^{2+}_o leads to cytoskeletal changes such as podosome assembly⁷² that may be linked to PLC activation and the associated rise in Ca^{2+}_i concentration⁷⁶. Release of high concentration of Ca^{2+} due to resorbing activity of the osteoclasts in turn inhibits bone resorption and/or increased osteoclast retraction and detachment from the bone⁷⁷. The mechanism of this feedback inhibition by calcium on osteoclast activity is not fully understood. Zaidi *et al*⁸⁰ detected an immunoreactive type II ryanodine receptor located in the osteoclastic plasma membrane and postulated that it might sense Ca^{2+}_o and regulate its influx⁸¹. However, high Ca^{2+}_o has been shown to inhibit osteoclast like cell formation by presumably acting on the CaR present in osteoclast precursor cells⁴⁹. Kameda *et al*⁵² have reported CaR expression in mature bone resorbing osteoclast isolated from rabbits. This group has also reported that the bone-resorbing activity of osteoclast, as determined by their pit formation, was inhibited by high Ca^{2+} as well as type I CaR agonists such as neomycin and sGd^{3+} .

Unequivocal evidence has recently been provided in support of CaR's role in both osteoclast differentiation and osteoclast apoptosis. Using CaR-*null* mice, Mentaverri *et al*⁸² have shown that osteoclast differentiation from the bone marrow precursor cells is 70 per cent less in CaR^{-/-} mice compared with CaR^{+/+} mice. On the other hand, mature osteoclasts undergo apoptosis in presence of high Ca^{2+} and a dominant negative CaR construct abrogates this effect indicating the role CaR in osteoclast apoptosis. The signaling pathways that are associated with osteoclast apoptosis by the CaR is likely mediated by PLC and NF- κ B (Fig. 4).

Role of calcium sensing receptor in haematopoietic stem cells

During mammalian ontogeny, haematopoietic stem cells (HSCs) translocate from the foetal liver to the bone marrow. Bone marrow is the site of haematopoiesis in adults. HSCs within the bone marrow cavity reside in close proximity to the endosteal surfaces of bone called 'stem cell niche'^{83,84}. It has been observed that transplanted HSCs migrate to these areas within hours of intravenous injection⁸⁵.

Several factors are involved in HSC homing at a specific site. Since its anatomical proximity to HSCs and that it produces many factors essential to the survival, renewal and maturation of HSCs, osteoblasts are the most important cell types determining HSC homing to bone marrow. These growth factors/cytokines include the colony-stimulating growth factors granulocyte-colony-stimulating factor (G-CSF), macrophage-colony-stimulating factor (M-CSF) and granulocyte/macrophage-colony-stimulating factor (GM-CSF), interleukin-1-beta (IL-1 β), interleukin-6 (IL-6), interleukin-7 (IL-7), osteoprotegerin, RANKL, stromal-derived factor-1, tumour-necrosis factor- α (TNF- α) and vascular endothelial growth factor (VEGF)⁸⁶. Furthermore, bone morphogenetic protein signaling that is crucial for osteoblast development and function is also involved in adult HSC development⁸⁷. In addition, angiopoietin-1 produced by osteoblasts activates the stem cell receptor tyrosine kinase Tie2 and thereby promotes tight adhesion of stem cells to their niche⁸⁸. Together, these observations strongly suggest that osteogenesis and haematopoiesis are functionally

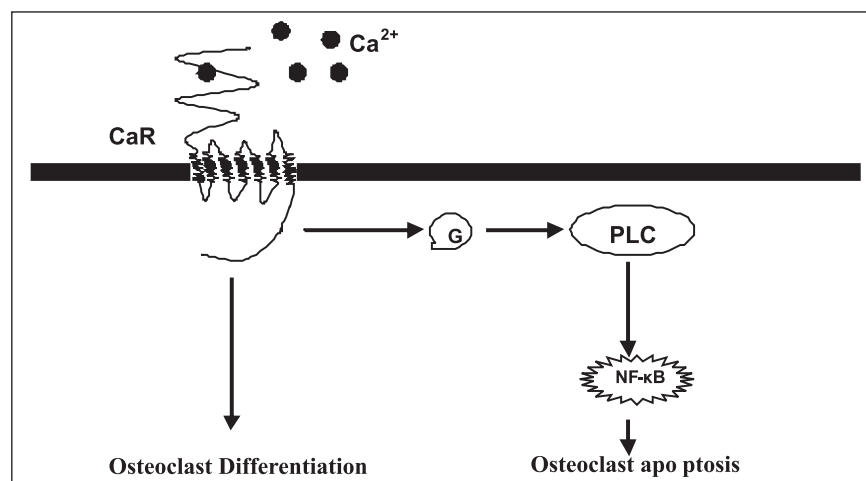


Fig. 4. Role of CaR in the differentiation and apoptosis of osteoclasts. Upon stimulation by Ca^{2+}_o , the CaR activates PLC resulting in the osteoclast differentiation. PLC activation by the CaR in turn will activate and translocate NF- κ B from the cytoplasm to the nucleus in mature osteoclasts along with some other transcriptional factors, which ultimately will lead to its apoptosis.

linked. Bone marrow stromal cells comprise the second major cell types providing appropriate environmental cues for haematopoiesis⁸⁹. The stroma is composed of a variety of stromal stem cells (SSCs), which are mostly derived from either mesenchymal or hematopoietic lineages. Finally, extracellular matrix proteins such as collagen and glycosamines, and adhesion molecules such as very late antigen-4 (VLA-4), very late antigen-5 (VLA-5), fibronectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) also influence HSC homing⁸⁶.

An essential prerequisite for the development of normal haematopoiesis in the bone marrow is endochondral ossification⁹⁰⁻⁹² which provides a crucial interrelationship between ossification and maturation of haematopoietic processes in mammals. A characteristic of the endosteum where active bone modelling and remodelling take place is an increased Ca^{2+} concentration, which reaches 40 mM near resorbing osteoclasts⁹³, a level that is about 33-fold more than serum calcium levels. CaR could be a putative target of this elevated levels of Ca^{2+} . Indeed, CaR expression has been shown on haematopoietic cells, including cells of the monocyte/macrophage lineage^{94,95}. CaR activation of these cells induces their transmigration *in vitro* and *in vivo*^{94,95}. Recent report by Adams *et al*⁹⁶ show that antenatal mice lacking *CaR* gene had primitive haematopoietic cells in the circulation and spleen, whereas a few were found in bone marrow. In addition, bone marrow of CaR deficient (*CaR*^{-/-}) mice exhibited hypocellularity and expanded extramedullary haematopoiesis. HSCs obtained from *CaR*^{-/-} mouse exhibited migration in response to stromal-derived growth factor-1 α gradients that is comparable with *CaR*^{+/+} mouse. No differences were observed in the number of *CaR*^{+/+} or *CaR*^{-/-} cells seeding the marrow space. However, *CaR*^{-/-} cells are unable to engage and remain within the niche, suggesting that CaR acts downstream of SDF-1 α chemotaxis. This defect could be due to the inability of the *CaR*^{-/-} cells to adhere to collagen I, which is the most abundant bone matrix protein produced by osteoblasts. Therefore, CaR could importantly participate in the stem cell behaviour within the bone marrow and modulate osseous homeostasis⁹⁶.

Roles for the calcium sensing receptor in malignancy induced osteolysis

As explained in the 'seed and soil' hypothesis, bone represents a fertile ground for cancer cells to flourish⁹⁶. Hence, humoral hypercalcaemia of malignancy (HHM) is one of the most frequent paraneoplastic syndromes

which include breast, prostate, lung, and renal carcinomas. HHM is a state in which serum calcium concentrations are typically greater than 12 mg/dl, corrected for serum albumin concentration. Several factors, for example, VEGF and interleukin-8 and -11, have been implicated in promoting hypercalcaemia of malignancy. However, PTHrP is considered to be the major pathogenic factor⁹⁷⁻⁹⁹ as the tumour burden and bone lesions have been shown to be decreased significantly by treatment with PTHrP-neutralizing antibody in mice inoculated with a human breast cancer cell line [MDA-MB-231]¹⁰⁰ or lung squamous cell carcinoma-derived cells [HARA]¹⁰¹. A clinical study reported that patients who develop metastatic breast disease in bone express PTHrP in >90 per cent of the cases while those who develop metastases in extraskelatal sites express PTHrP in only 17 per cent of cases, suggesting that PTHrP plays a key role in the pathogenesis of skeletal metastases¹⁰². When produced in excess by extraskelatal tumour cells as in the case of HHM, PTHrP spills into the systemic circulation and acts on the same PTH receptor that mediates the Ca^{2+} elevating actions of PTH on bone and kidney. The resultant hypercalcaemia can rapidly become severe and life threatening.

Bone is the depot for calcium in the body. One of the direct effects of increased bone resorption during HHM is an increase in Ca^{2+} . Ca^{2+} released during the resorptive process into the bony microenvironment could modulate the normal remodelling process as well as metastatic osteolysis. In particular, large changes in local Ca^{2+} due to Ca^{2+} release could be "sensed" by the cancer cells as well as nearby cells, such as osteoblasts, osteoclasts, and monocytes. High Ca^{2+} stimulates PTHrP production in both normal^{103,104} and malignant cells^{105,106}. CaR could enable Ca^{2+} to contribute directly to this vicious cycle by stimulating the production of PTHrP by the cancer cells instead of being a mere by-product of these malignancies². In that case, the CaR could serve as a central element in a physiologically inappropriate "feed-forward" mechanism, whereby malignant osteolysis would induce further osteolysis. CaR-stimulated secretion of PTHrP could afford a selective advantage by contributing to tumour cell survival and growth and/or skeletal complications (*e.g.*, osteolysis), as PTHrP has been shown to promote growth and survival of tumours that secrete it¹⁰⁷.

CaR activation has been shown to stimulate PTHrP secretion from human breast cancer (MCF-7, MDA-MB-231)¹⁰⁸, prostate cancer (PC-3 and LnCaP)¹⁰⁹ and

rat H-500 Leydig cells¹⁰⁸. In addition, CaR activation has been shown to stimulate proliferation of H-500 cells as well as protects the cells from serum-induced apoptosis¹¹⁰. PTHrP secretion in these various cell types are regulated by PKC-dependent or -independent activation of MEK/ERK and p38 MAP kinase pathways^{107,111-114}. In addition, CaR has been shown to transactivate the epidermal growth factor receptor pathway in prostate and H-500 cells¹¹⁵⁻¹¹⁸. CaR has also been shown to activate a pro-survival PKB/PI3 kinase/AKT pathway in H-500 cells¹¹⁹. Finally, CaR activation has been shown to promote angiogenic genes such as pituitary tumour transforming gene and vascular endothelial growth factor^{120,121}. Taken together, CaR stimulation appears to have a tumour promoting roles in various models of HHM cell types. Whether these observations are true *in vivo* will determine if specific CaR antagonist (so called calcilytic) could be used therapeutically.

Conclusion

Significant advances have been made in understanding the roles of the CaR in the various bone cells. Activating the CaR serves two highly desirable functions, *i.e.*, activating the osteoblasts (bone anabolic effect) and inducing the apoptosis of osteoclasts (anti-resorption). Therefore, CaR could be a therapeutic target for developing dual action drug for bone loss disorders such as osteoporosis. Indeed, strontium ranelate, the only dual action drug that has recently been launched acts by activating the CaR in osteoblasts and in osteoclasts (personal communication Dr R. Mentaverri). CaR also may play an important role in HHM as a mediator of a malignancy-associated, feed forward loop between the tumour and bone, resulting in osteolysis. Whether, antagonizing the CaR by calcilytic could be of therapeutic potential awaits *in vivo* documentation of the tumour promoting roles of the CaR in HHM.

Acknowledgment

The work was supported in part by a NIH grant (AR-02215) to NC.

References

1. Brown EM. Extracellular Ca²⁺ sensing, regulation of parathyroid cell function, and role of Ca²⁺ and other ions as extracellular (first) messengers. *Physiol Rev* 1991; 71 : 371-411.
2. Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, *et al.* Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* 1993; 366 : 575-80.
3. Brown EM, MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. *Physiol Rev* 2001; 81 : 239-97.
4. Riccardi D, Park J, Lee WS, Gamba G, Brown EM, Hebert SC. Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. *Proc Natl Acad Sci USA* 1995; 92 : 131-5.
5. Brown EM. Four-parameter model of the sigmoidal relationship between parathyroid hormone release and extracellular calcium concentration in normal and abnormal parathyroid tissue. *J Clin Endocrinol Metab* 1983; 56 : 572-81.
6. Nemeth EF, Scarpa A. Cytosolic Ca²⁺ and the regulation of secretion in parathyroid cells. *FEBS Lett* 1986; 203 : 15-9.
7. Nemeth EF, Scarpa A. Rapid mobilization of cellular Ca²⁺ in bovine parathyroid cells evoked by extracellular divalent cations. Evidence for a cell surface calcium receptor. *J Biol Chem* 1987; 262 : 5188-96.
8. Nemeth EF, Kosz LM. Adenine nucleotides mobilize cellular Ca²⁺ and inhibit parathyroid hormone secretion. *Am J Physiol* 1989; 257 : E505-13.
9. Cima RR, Cheng I, Klingensmith ME, Chattopadhyay N, Kifor O, Hebert SC, *et al.* Identification and functional assay of an extracellular calcium-sensing receptor in Necturus gastric mucosa. *Am J Physiol* 1997; 273 : G1051-60.
10. Ruat M, Molliver ME, Snowman AM, Snyder SH. Calcium sensing receptor: molecular cloning in rat and localization to nerve terminals. *Proc Natl Acad Sci USA* 1995; 92 : 3161-5.
11. Garrett JE, Capuano IV, Hammerland LG, Hung BC, Brown EM, Hebert SC, *et al.* Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. *J Biol Chem* 1995; 270 : 12919-25.
12. Bai M, Trivedi S, Brown EM. Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. *J Biol Chem* 1998; 273 : 23605-10.
13. Ward DT, Brown EM, Harris HW. Disulfide bonds in the extracellular calcium-polyvalent cation-sensing receptor correlate with dimer formation and its response to divalent cations *in vitro*. *J Biol Chem* 1998; 273 : 14476-83.
14. Hofer AM, Brown EM. Extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol* 2003; 4 : 530-8.
15. Brown EM. Physiology and pathophysiology of the extracellular calcium-sensing receptor. *Am J Med* 1999; 106 : 238-53.
16. Pollak MR, Brown EM, Chou YH, Hebert SC, Marx SJ, Steinmann B, *et al.* Mutations in the human Ca(2+)-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell* 1993; 75 : 1297-303.
17. Pollak MR, Brown EM, Estep HL, McLaine PN, Kifor O, Park J, *et al.* Autosomal dominant hypocalcaemia caused by a Ca(2+)-sensing receptor gene mutation. *Nat Genet* 1994; 8 : 303-7.
18. Brown EM. Familial hypocalciuric hypercalcemia and other disorders with resistance to extracellular calcium. *Endocrinol Metab Clin North Am* 2000; 29 : 503-22.

19. Foley TP Jr, Harrison HC, Arnaud CD, Harrison HE. Familial benign hypercalcemia. *J Pediatr* 1972; 81 : 1060-7.
20. Marx SJ, Attie MF, Levine MA, Spiegel AM, Downs RW Jr, Lasker RD. The hypocalciuric or benign variant of familial hypercalcemia: clinical and biochemical features in fifteen kindreds. *Medicine (Baltimore)* 1981; 60 : 397-412.
21. Law WM Jr, Heath H 3rd. Familial benign hypercalcemia (hypocalciuric hypercalcemia). Clinical and pathogenetic studies in 21 families. *Ann Intern Med* 1985; 102 : 511-9.
22. Heath H 3rd, Jackson CE, Otterud B, Leppert MF. Genetic linkage analysis in familial benign (hypocalciuric) hypercalcemia: evidence for locus heterogeneity. *Am J Hum Genet* 1993; 53 : 193-200.
23. Davies M, Klimiuk PS, Adams PH, Lumb GA, Large DM, Anderson DC. Familial hypocalciuric hypercalcaemia and acute pancreatitis. *Br Med J (Clin Res Ed)* 1981; 282 : 1023-5.
24. Bai M, Janicic N, Trivedi S, Quinn SJ, Cole DE, Brown EM, *et al.* Markedly reduced activity of mutant calcium-sensing receptor with an inserted Alu element from a kindred with familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *J Clin Invest* 1997; 99 : 1917-25.
25. Heath H, 3rd. Familial benign hypercalcemia-from clinical description to molecular genetics. *West J Med* 1994; 160 : 554-61.
26. Pearce S, Steinmann B. Casting new light on the clinical spectrum of neonatal severe hyperparathyroidism. *Clin Endocrinol (Oxf)* 1999; 50 : 691-3.
27. Marx SJ, Lasker RD, Brown EM, Fitzpatrick LA, Sweezey NB, Goldbloom RB, *et al.* Secretory dysfunction in parathyroid cells from a neonate with severe primary hyperparathyroidism. *J Clin Endocrinol Metab* 1986; 62 : 445-9.
28. Pollak MR, Chou YH, Marx SJ, Steinmann B, Cole DE, Brandi ML, *et al.* Familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. Effects of mutant gene dosage on phenotype. *J Clin Invest* 1994; 93 : 1108-12.
29. Kobayashi M, Tanaka H, Tsuzuki K, Tsuyuki M, Igaki H, Ichinose Y, *et al.* Two novel missense mutations in calcium-sensing receptor gene associated with neonatal severe hyperparathyroidism. *J Clin Endocrinol Metab* 1997; 82 : 2716-9.
30. Bai M, Pearce SH, Kifor O, Trivedi S, Stauffer UG, Thakker RV, *et al.* *In vivo* and *in vitro* characterization of neonatal hyperparathyroidism resulting from a *de novo*, heterozygous mutation in the Ca²⁺-sensing receptor gene: normal maternal calcium homeostasis as a cause of secondary hyperparathyroidism in familial benign hypocalciuric hypercalcemia. *J Clin Invest* 1997; 99 : 88-96.
31. De Luca F, Ray K, Mancilla EE, Fan GF, Winer KK, Gore P, *et al.* Sporadic hypoparathyroidism caused by *de novo* gain-of-function mutations of the Ca(2⁺)-sensing receptor. *J Clin Endocrinol Metab* 1997; 82 : 2710-5.
32. Baron J, Winer KK, Yanovski JA, Cunningham AW, Laue L, Zimmerman D, *et al.* Mutations in the Ca(2⁺)-sensing receptor gene cause autosomal dominant and sporadic hypoparathyroidism. *Hum Mol Genet* 1996; 5 : 601-6.
33. Lovlie R, Eiken HG, Sorheim JI, Boman H. The Ca(2+)-sensing receptor gene (PCAR1) mutation T151M in isolated autosomal dominant hypoparathyroidism. *Hum Genet* 1996; 98 : 129-33.
34. Ho C, Conner DA, Pollak MR, Ladd DJ, Kifor O, Warren HB, *et al.* A mouse model of human familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Nat Genet* 1995; 11 : 389-94.
35. Ray K, Northup J. Evidence for distinct cation and calcimimetic compound (NPS 568) recognition domains in the transmembrane regions of the human Ca²⁺ receptor. *J Biol Chem* 2002; 277 : 18908-13.
36. Nemeth EF, Heaton WH, Miller M, Fox J, Balandrin MF, Van Wagenen BC, *et al.* Pharmacodynamics of the type II calcimimetic compound cinacalcet HCl. *J Pharmacol Exp Ther* 2004; 308 : 627-35.
37. Nemeth EF, Delmar EG, Heaton WL, Miller MA, Lambert LD, Conklin RL, *et al.* Calcilytic compounds: potent and selective Ca²⁺ receptor antagonists that stimulate secretion of parathyroid hormone. *J Pharmacol Exp Ther* 2001; 299 : 323-31.
38. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, *et al.* Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med* 2001; 344 : 1434-41.
39. Dvorak MM, Riccardi D. Ca²⁺ as an extracellular signal in bone. *Cell Calcium* 2004; 35 : 249-55.
40. Sugimoto T, Kanatani M, Kano J, Kaji H, Tsukamoto T, Yamaguchi T, *et al.* Effects of high calcium concentration on the functions and interactions of osteoblastic cells and monocytes and on the formation of osteoclast-like cells. *J Bone Miner Res* 1993; 8 : 1445-52.
41. Fujikawa Y, Quinn JM, Sabokbar A, McGee JO, Athanasou NA. The human osteoclast precursor circulates in the monocyte fraction. *Endocrinology* 1996; 137 : 4058-60.
42. Quarles LD, Hartle JE, 2nd, Siddhanti SR, Guo R, Hinson TK. A distinct cation-sensing mechanism in MC3T3-E1 osteoblasts functionally related to the calcium receptor. *J Bone Miner Res* 1997; 12 : 393-402.
43. Yamaguchi T, Chattopadhyay N, Kifor O, Butters RR, Jr, Sugimoto T, Brown EM. Mouse osteoblastic cell line (MC3T3-E1) expresses extracellular calcium (Ca²⁺)-sensing receptor and its agonists stimulate chemotaxis and proliferation of MC3T3-E1 cells. *J Bone Miner Res* 1998; 13 : 1530-8.
44. Godwin SL, Soltoff SP. Extracellular calcium and platelet-derived growth factor promote receptor-mediated chemotaxis in osteoblasts through different signaling pathways. *J Biol Chem* 1997; 272 : 11307-12.
45. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol* 1983; 96 : 191-8.
46. Fukumoto S. Localization and function of calcium-sensing mechanism in bone cells. *Nippon Rinsho* 1998; 56 : 1419-24.
47. Chang W, Tu C, Chen TH, Komuves L, Oda Y, Pratt SA, *et al.* Expression and signal transduction of calcium-sensing receptors in cartilage and bone. *Endocrinology* 1999; 140 : 5883-93.
48. Takeyama S, Yoshimura Y, Shirai Y, Deyama Y, Hasegawa T, Yawaka Y, *et al.* Low calcium environment effects

- osteoprotegerin ligand/osteoclast differentiation factor. *Biochem Biophys Res Commun* 2000; 276 : 524-9.
49. Kanatani M, Sugimoto T, Kanzawa M, Yano S, Chihara K. High extracellular calcium inhibits osteoclast-like cell formation by directly acting on the calcium-sensing receptor existing in osteoclast precursor cells. *Biochem Biophys Res Commun* 1999; 261 : 144-8.
 50. Yamaguchi T, Kifor O, Chattopadhyay N, Bai M, Brown EM. Extracellular calcium (Ca²⁺o)-sensing receptor in a mouse monocyte-macrophage cell line (J774): potential mediator of the actions of Ca²⁺o on the function of J774 cells. *J Bone Miner Res* 1998; 13 : 1390-7.
 51. Yamaguchi T, Ye C, Chattopadhyay N, Sanders JL, Vassilev PM, Brown EM. Enhanced expression of extracellular calcium sensing receptor in monocyte-differentiated versus undifferentiated HL-60 cells: potential role in regulation of a nonselective cation channel. *Calcif Tissue Int* 2000; 66 : 375-82.
 52. Kameda T, Mano H, Yamada Y, Takai H, Amizuka N, Kobori M, *et al.* Calcium-sensing receptor in mature osteoclasts, which are bone resorbing cells. *Biochem Biophys Res Commun* 1998; 245 : 419-22.
 53. Mailland M, Waelchli R, Ruat M, Boddeke HG, Seuwen K. Stimulation of cell proliferation by calcium and a calcimimetic compound. *Endocrinology* 1997; 138 : 3601-5.
 54. Yamaguchi T, Kifor O, Chattopadhyay N, Brown EM. Expression of extracellular calcium (Ca²⁺ + o)-sensing receptor in the clonal osteoblast-like cell lines, UMR-106 and SAOS-2. *Biochem Biophys Res Commun* 1998; 243 : 753-7.
 55. Yamaguchi T, Chattopadhyay N, Kifor O, Ye C, Vassilev PM, Sanders JL, *et al.* Expression of extracellular calcium-sensing receptor in human osteoblastic MG-63 cell line. *Am J Physiol Cell Physiol* 2001; 280 : C382-93.
 56. Pi M, Quarles LD. Osteoblast calcium-sensing receptor has characteristics of ANF/7TM receptors. *J Cell Biochem* 2005; 95 : 1081-92.
 57. Pi M, Faber P, Ekema G, Jackson PD, Ting A, Wang N, *et al.* Identification of a novel extracellular cation-sensing G-protein-coupled receptor. *J Biol Chem* 2005; 280 : 40201-9.
 58. Chattopadhyay N, Yano S, Tfelt-Hansen J, Rooney P, Kanuparthi D, Bandyopadhyay S, *et al.* Mitogenic action of calcium-sensing receptor on rat calvarial osteoblasts. *Endocrinology* 2004; 145 : 3451-62.
 59. Ward BK, Magno AL, Davis EA, Hanyaloglu AC, Stuckey BG, Burrows M, *et al.* Functional deletion of the calcium-sensing receptor in a case of neonatal severe hyperparathyroidism. *J Clin Endocrinol Metab* 2004; 89 : 3721-30.
 60. Chattopadhyay N, Quinn SJ, Kifor O, Ye C, Brown EM. The calcium-sensing receptor (CaR) is involved in strontium ranelate-induced osteoblast proliferation. *Biochem Pharmacol* 2007; 74 : 438-47.
 61. Hartle JE, 2nd, Prpic V, Siddhanti SR, Spurney RF, Quarles LD. Differential regulation of receptor-stimulated cyclic adenosine monophosphate production by polyvalent cations in MC3T3-E1 osteoblasts. *J Bone Miner Res* 1996; 11 : 789-99.
 62. Yamaguchi T, Chattopadhyay N, Kifor O, Sanders JL, Brown EM. Activation of p42/44 and p38 mitogen-activated protein kinases by extracellular calcium-sensing receptor agonists induces mitogenic responses in the mouse osteoblastic MC3T3-E1 cell line. *Biochem Biophys Res Commun* 2000; 279 : 363-8.
 63. Ye CP, Yamaguchi T, Chattopadhyay N, Sanders JL, Vassilev PM, Brown EM. Extracellular calcium-sensing-receptor (CaR)-mediated opening of an outward K(+) channel in murine MC3T3-E1 osteoblastic cells: evidence for expression of a functional CaR. *Bone* 2000; 27 : 21-7.
 64. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, *et al.* Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997; 89 : 309-19.
 65. Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T, *et al.* Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem Biophys Res Commun* 1997; 234 : 137-42.
 66. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinoshita M, Mochizuki S, *et al.* Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 1998; 95 : 3597-602.
 67. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, *et al.* Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998; 93 : 165-76.
 68. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, *et al.* Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999; 402 : 304-9.
 69. Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, *et al.* Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci USA* 1999; 96 : 3540-5.
 70. Datta HK, MacIntyre I, Zaidi M. Intracellular calcium in the control of osteoclast function. I. Voltage-insensitivity and lack of effects of nifedipine, BAYK8644 and diltiazem. *Biochem Biophys Res Commun* 1990; 167 : 183-8.
 71. Zaidi M, MacIntyre I, Datta H. Intracellular calcium in the control of osteoclast function. II. Paradoxical elevation of cytosolic free calcium by verapamil. *Biochem Biophys Res Commun* 1990; 167 : 807-12.
 72. Miyauchi A, Hruska KA, Greenfield EM, Duncan R, Alvarez J, Barattolo R, *et al.* Osteoclast cytosolic calcium, regulated by voltage-gated calcium channels and extracellular calcium, controls podosome assembly and bone resorption. *J Cell Biol* 1990; 111 : 2543-52.
 73. Shankar VS, Alam AS, Bax CM, Bax BE, Pazianas M, Huang CL, *et al.* Activation and inactivation of the osteoclast Ca²⁺ receptor by the trivalent cation, La³⁺. *Biochem Biophys Res Commun* 1992; 187 : 907-12.
 74. Zaidi M, Kerby J, Huang CL, Alam T, Rathod H, Chambers TJ, *et al.* Divalent cations mimic the inhibitory effect of extracellular ionised calcium on bone resorption by isolated rat osteoclasts: further evidence for a "calcium receptor". *J Cell Physiol* 1991; 149 : 422-7.
 75. Shankar VS, Bax CM, Alam AS, Bax BE, Huang CL, Zaidi M. The osteoclast Ca²⁺ receptor is highly sensitive to

- activation by transition metal cations. *Biochem Biophys Res Commun* 1992; 187 : 913-8.
76. Zaidi M, Datta HK, Patchell A, Moonga B, MacIntyre I. 'Calcium-activated' intracellular calcium elevation: a novel mechanism of osteoclast regulation. *Biochem Biophys Res Commun* 1989; 163 : 1461-5.
 77. Datta HK, MacIntyre I, Zaidi M. The effect of extracellular calcium elevation on morphology and function of isolated rat osteoclasts. *Biosci Rep* 1989; 9 : 747-51.
 78. Moonga BS, Moss DW, Patchell A, Zaidi M. Intracellular regulation of enzyme secretion from rat osteoclasts and evidence for a functional role in bone resorption. *J Physiol* 1990; 429 : 29-45.
 79. Boonkamp PM, van der Wee-Pals LJ, van Wijk-van Lennep MM, Thesing CW, Bijvoet OL. Two modes of action of bisphosphonates on osteoclastic resorption of mineralized matrix. *Bone Miner* 1986; 1 : 27-39.
 80. Zaidi M, Shankar VS, Tunwell R, Adebajo OA, Mackrill J, Pazianas M, *et al.* A ryanodine receptor-like molecule expressed in the osteoclast plasma membrane functions in extracellular Ca²⁺ sensing. *J Clin Invest* 1995; 96 : 1582-90.
 81. Zaidi M, Adebajo OA, Moonga BS, Sun L, Huang CL. Emerging insights into the role of calcium ions in osteoclast regulation. *J Bone Miner Res* 1999; 14 : 669-74.
 82. Mentaverri R, Yano S, Chattopadhyay N, Petit L, Kifor O, Kamel S, *et al.* The calcium sensing receptor is directly involved in both osteoclast differentiation and apoptosis. *FASEB J* 2006; 20 : 2562-4.
 83. Lord BI, Testa NG, Hendry JH. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood* 1975; 46 : 65-72.
 84. Gong JK. Endosteal marrow: a rich source of hematopoietic stem cells. *Science* 1978; 199 : 1443-5.
 85. Nilsson SK, Johnston HM, Coverdale JA. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood* 2001; 97 : 2293-9.
 86. Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood* 2005; 105 : 2631-9.
 87. Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 2003; 425 : 836-41.
 88. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 2004; 118 : 149-61.
 89. Kuznetsov SA, Riminucci M, Ziran N, Tsutsui TW, Corsi A, Calvi L, *et al.* The interplay of osteogenesis and hematopoiesis: expression of a constitutively active PTH/PTHrP receptor in osteogenic cells perturbs the establishment of hematopoiesis in bone and of skeletal stem cells in the bone marrow. *J Cell Biol* 2004; 167 : 1113-22.
 90. Zhou H, Choong PF, Henderson S, Chou ST, Aspenberg P, Martin TJ, *et al.* Marrow development and its relationship to bone formation *in vivo*: a histological study using an implantable titanium device in rabbits. *Bone* 1995; 17 : 407-15.
 91. Deguchi K, Yagi H, Inada M, Yoshizaki K, Kishimoto T, Komori T. Excessive extramedullary hematopoiesis in Cbfa1-deficient mice with a congenital lack of bone marrow. *Biochem Biophys Res Commun* 1999; 255 : 352-9.
 92. Jacenko O, Roberts DW, Campbell MR, McManus PM, Gress CJ, Tao Z. Linking hematopoiesis to endochondral skeletogenesis through analysis of mice transgenic for collagen X. *Am J Pathol* 2002; 160 : 2019-34.
 93. Silver IA, Murrills RJ, Etherington DJ. Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. *Exp Cell Res* 1988; 175 : 266-76.
 94. House MG, Kohlmeier L, Chattopadhyay N, Kifor O, Yamaguchi T, Leboff MS, *et al.* Expression of an extracellular calcium-sensing receptor in human and mouse bone marrow cells. *J Bone Miner Res* 1997; 12 : 1959-70.
 95. Olszak IT, Poznansky MC, Evans RH, Olson D, Kos C, Pollak MR, *et al.* Extracellular calcium elicits a chemokinetic response from monocytes *in vitro* and *in vivo*. *J Clin Invest* 2000; 105 : 1299-305.
 96. Paget S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev* 1989; 8 : 98-101.
 97. Grill V, Ho P, Body JJ, Johanson N, Lee SC, Kukreja SC, *et al.* Parathyroid hormone-related protein: elevated levels in both humoral hypercalcemia of malignancy and hypercalcemia complicating metastatic breast cancer. *J Clin Endocrinol Metab* 1991; 73 : 1309-15.
 98. Stewart AF, Horst R, Deftos LJ, Cadman EC, Lang R, Broadus AE. Biochemical evaluation of patients with cancer-associated hypercalcemia: evidence for humoral and nonhumoral groups. *N Engl J Med* 1980; 303 : 1377-83.
 99. Suva LJ, Winslow GA, Wettenhall RE, Hammonds RG, Moseley JM, Diefenbach-Jagger H, *et al.* A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science* 1987; 237 : 893-6.
 100. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, *et al.* Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest* 1996; 98 : 1544-9.
 101. Iguchi H, Tanaka S, Ozawa Y, Kashiwakuma T, Kimura T, Hiraga T, *et al.* An experimental model of bone metastasis by human lung cancer cells: the role of parathyroid hormone-related protein in bone metastasis. *Cancer Res* 1996; 56 : 4040-3.
 102. Powell GJ, Southby J, Danks JA, Stillwell RG, Hayman JA, Henderson MA, *et al.* Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. *Cancer Res* 1991; 51 : 3059-61.
 103. Brandt DW, Pandol SJ, Deftos LJ. Calcium-stimulated parathyroid hormone-like protein secretion: potentiation through a protein kinase-C pathway. *Endocrinology* 1991; 128 : 2999-3004.
 104. Kremer R, Woodworth CD, Goltzman D. Expression and action of parathyroid hormone-related peptide in human cervical epithelial cells. *Am J Physiol* 1996; 271 : C164-71.
 105. Merryman JI, Capen CC, McCauley LK, Werkmeister JR, Suter MM, Rosol TJ. Regulation of parathyroid hormone-

- related protein production by a squamous carcinoma cell line *in vitro*. *Lab Invest* 1993; 69 : 347-54.
106. Rizzoli R, Feyen JH, Grau G, Wohlwend A, Sappino AP, Bonjour JP. Regulation of parathyroid hormone-related protein production in a human lung squamous cell carcinoma line. *J Endocrinol* 1994; 143 : 333-41.
107. Schramek H. MAP kinases: from intracellular signals to physiology and disease. *News Physiol Sci* 2002; 17 : 62-7.
108. Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Butters RR, Brown EM. Extracellular calcium-sensing receptor expression and its potential role in regulating parathyroid hormone-related peptide secretion in human breast cancer cell lines. *Endocrinology* 2000; 141 : 4357-64.
109. Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Brown EM. Ca(2+)-sensing receptor expression and PTHrP secretion in PC-3 human prostate cancer cells. *Am J Physiol Endocrinol Metab* 2001; 281 : E1267-74.
110. Tfelt-Hansen J, Chattopadhyay N, Yano S, Kanuparthi D, Rooney P, Schwarz P, *et al*. Calcium-sensing receptor induces proliferation through p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase but not extracellularly regulated kinase in a model of humoral hypercalcemia of malignancy. *Endocrinology* 2004; 145 : 1211-7.
111. Awata H, Huang C, Handlogten ME, Miller RT. Interaction of the calcium-sensing receptor and filamin, a potential scaffolding protein. *J Biol Chem* 2001; 276 : 34871-9.
112. Hjalms G, MacLeod RJ, Kifor O, Chattopadhyay N, Brown EM. Filamin-A binds to the carboxyl-terminal tail of the calcium-sensing receptor, an interaction that participates in CaR-mediated activation of mitogen-activated protein kinase. *J Biol Chem* 2001; 276 : 34880-7.
113. Keller ET, Brown J. Prostate cancer bone metastases promote both osteolytic and osteoblastic activity. *J Cell Biochem* 2004; 91 : 718-29.
114. MacLeod RJ, Chattopadhyay N, Brown EM. PTHrP stimulated by the calcium-sensing receptor requires MAP kinase activation. *Am J Physiol Endocrinol Metab* 2003; 284 : E435-42.
115. MacLeod RJ, Yano S, Chattopadhyay N, Brown EM. Extracellular calcium-sensing receptor transactivates the epidermal growth factor receptor by a triple-membrane-spanning signaling mechanism. *Biochem Biophys Res Commun* 2004; 320 : 455-60.
116. Tfelt-Hansen J, Yano S, John Macleod R, Smajilovic S, Chattopadhyay N, Brown EM. High calcium activates the EGF receptor potentially through the calcium-sensing receptor in Leydig cancer cells. *Growth Factors* 2005; 23 : 117-23.
117. Tomlins SA, Bollinger N, Creim J, Rodland KD. Cross-talk between the calcium-sensing receptor and the epidermal growth factor receptor in Rat-1 fibroblasts. *Exp Cell Res* 2005; 308 : 439-45.
118. Yano S, Macleod RJ, Chattopadhyay N, Tfelt-Hansen J, Kifor O, Butters RR, *et al*. Calcium-sensing receptor activation stimulates parathyroid hormone-related protein secretion in prostate cancer cells: role of epidermal growth factor receptor transactivation. *Bone* 2004; 35 : 664-72.
119. Tfelt-Hansen J, MacLeod RJ, Chattopadhyay N, Yano S, Quinn S, Ren X, *et al*. Calcium-sensing receptor stimulates PTHrP release by pathways dependent on PKC, p38 MAPK, JNK, and ERK1/2 in H-500 cells. *Am J Physiol Endocrinol Metab* 2003; 285 : E329-37.
120. Pei L, Melmed S. Isolation and characterization of a pituitary tumor-transforming gene (PTTG). *Mol Endocrinol* 1997; 11 : 433-41.
121. Ishikawa H, Heaney AP, Yu R, Horwitz GA, Melmed S. Human pituitary tumor-transforming gene induces angiogenesis. *J Clin Endocrinol Metab* 2001; 86 : 867-74.
122. Horwitz MJ, Stewart AF. Humoral hypercalcemia of malignancy. In: *Primer on the metabolic bone diseases and disorders of mineral metabolism*. Favus MJ, editor. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2003. p. 246-50.

Reprint requests: Dr Naibedya Chattopadhyay, Division of Endocrinology, Central Drug Research Institute, Chattar Manzil Place
P.O. Box 173, Lucknow 223 001, India
e-mail: n_chattopadhyay@cdri.res.in