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THE ACTIVITY OF PEPTIDES OF THE CALCITONIN FAMILY IN BONE

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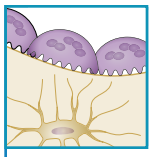

Naot D, Musson DS, Cornish J. The Activity of Peptides of the Calcitonin Family in Bone. *Physiol Rev* 99: 781–805, 2019. Published December 12, 2018; doi: 10.1152/physrev.00066.2017.—Calcitonin was discovered over 50 yr ago as a new hormone that rapidly lowers circulating calcium levels. This effect is caused by the inhibition of calcium efflux from bone, as calcitonin is a potent inhibitor of bone resorption. Calcitonin has been in clinical use for conditions of accelerated bone turnover, including Paget's disease and osteoporosis; although in recent years, with the development of drugs that are more potent inhibitors of bone resorption, its use has declined. A number of peptides that are structurally similar to calcitonin form the calcitonin family, which currently includes calcitonin gene–related peptides (αCGRP and βCGRP), amylin, adrenomedullin, and intermedin. Apart from being structurally similar, the peptides signal through related receptors and have some overlapping biological activities, although other activities are peptide specific. In bone, in vitro studies and administration of the peptides to animals generally found inhibitory effects on osteoclasts and bone resorption and positive effects on osteoblasts and bone formation. Surprisingly, studies in genetically modified mice have demonstrated that the physiological role of calcitonin appears to be the inhibition of osteoblast activity and bone turnover, whereas amylin inhibits osteoclast activity. The review article focuses on the activities of peptides of the calcitonin family in bone and the challenges in understanding the relationship between the pharmacological effects and the physiological roles of these peptides.

I. INTRODUCTION

A. The Calcitonin Family of Peptides

Calcitonin is a peptide hormone secreted from the parafollicular cells of the thyroid gland in response to an increase in serum calcium. The calcitonin family of peptides includes a number of peptide hormones that are structurally related to calcitonin and signal through common receptors, although they are expressed in different tissues and respond to different signals. Although some of the biological activities of the family members overlap, others are unique, and the main physiological functions of the peptide hormones are diverse.

1. Calcitonin

Calcium homeostasis is mainly controlled by the skeleton, the gut, and the kidney. The coordinated actions of these organs maintain the circulating concentrations of calcium within the narrow range of 8.5 and 10.5 mg/dL (2.12–2.62 mM). The tight control of circulating calcium levels is essential for major physiological processes, including muscle contraction, neuronal excitation, glycogen metabolism, and coagulation. At the cellular level, calcium ions play key roles in basic functions, such as cell adhesion and division (69). The importance of calcium homeostasis and the detrimental consequences of calcium imbalance have been recognized by physicians for hundreds of years.

The discovery of the parathyroid glands in the late 19th century was central to the understanding of the hormonal mechanisms that control calcium homeostasis (168). The physiological function of the gland was described later, in 1925, when Collip (39) showed that acid extracts of the parathyroid gland can reverse the tetany caused by parathyroidectomy. He concluded that the gland is an endocrine organ that secretes a parathyroid hormone (PTH), which responds to low calcium levels and acts to restore them back to the normal range. With the improvement of methods for PTH extraction, the mechanism of action of PTH could be further investigated, and it was found that the hormone mobilizes calcium from bone (13, 147).

Calcium homeostasis, and in particular the role of the thyroid–parathyroid in the regulation of calcium levels, was investigated by Copp and Cheney in a perfusion system in

anesthetized dogs (42). Perfusion of the thyroid parathyroid glands with high levels of calcium caused a rapid fall (within 15 min) in the level of calcium in the blood. As PTH was the only hormone known to be secreted from the gland, the researchers hypothesized that the perfused high calcium concentrations suppressed PTH secretion, which, in turn, caused the observed fall in systemic calcium levels. To test this hypothesis, the investigators removed the thyroid–parathyroid from the dogs, expecting the same effect on circulating calcium levels in the absence of PTH. However, to their surprise, systemic calcium levels remained high. They concluded that hypercalcemia does not merely suppress the production of PTH, but stimulates the production of a hormone that lowers calcium levels in the blood (41, 42). The name "calcitonin" was suggested for the putative agent that controls calcium tone. Subsequent studies established that calcitonin is produced by the thyroid gland, and a number of investigators suggested the alternative name "thyrocalcitonin" (95).

Biological assays that had been developed to investigate of the mechanisms of action of PTH in controlling circulating calcium levels were used to study calcitonin. Using one of these experimental systems, Friedman and Raisz (73) injected pregnant rats with ^{45}Ca and then placed embryonic bone in tissue culture in a bioassay used to determine the release of radioactively labeled calcium as a measure of bone resorption. The effects of partially purified calcitonin from extracts of rat thyroid gland were measured in baseline conditions and in combination with PTH, which induced bone resorption. The results of the study showed that calcitonin inhibits both basal and PTH-stimulated bone resorption, thus identifying the underlying mechanism of calcitonin's hypocalcemic effect (73). Further evidence for the direct inhibition of bone resorption by calcitonin came from an in vivo study that measured the collagen breakdown product hydroxyproline in urine of rats treated with highly purified porcine calcitonin (142). The study showed that calcitonin rapidly diminished urinary hydroxyproline excretion, indicating direct inhibition of bone resorption and collagen breakdown.

Calcitonin was subsequently purified from several species: mammals, birds, and fish (230). Purification of calcitonin from human thyroid gland proved to be challenging, because it is produced at very low levels. Human calcitonin (hCT) was eventually purified by Neher et al. (161) from patients with tumors of the C cells of the thyroid, who produced high levels of calcitonin. The study determined the amino acid sequence of hCT and found considerable variability between pig calcitonin and hCT, although both are 32–amino acid peptides that contain a disulfide bridge near the amino-terminal and have an amidated carboxy-terminal. Linkage analysis mapped the hCT gene *CALCA* to the short arm of chromosome 11 (122). Although C cells of the thyroid are undoubtedly the major source of circulating calcitonin in humans, calcitonin-like immunoreactivity has also been identified in the prostate gland, the gastrointestinal tract, thymus, bladder, lung, and central nervous system (70). Interestingly, there is evidence to suggest the presence of calcitonin-like immunoreactivity in blood and urine samples from patients who had total thyroidectomy, indicating extrathyroidal secretion of calcitonin.

2. Calcitonin gene–related peptide

The organization of eukaryotic genes in exons and intervening "silent" introns, which are cleaved in the process of RNA maturation, was discovered simultaneously by a number of groups (76). Numerous studies demonstrating alternative splicing as a mechanism of post-transcriptional regulation followed this discovery. In a study of rat calcitonin-producing medullary thyroid carcinoma line, Rosenfeld et al. (180) found that in the process of serial transplantation of the carcinoma cell line, some of the tumors changed their phenotype and produced only very low levels of calcitonin. Analysis of these cell lines showed that with the change into low or no production of calcitonin, a new mRNA species, larger than calcitonin mRNA, appeared in the cytoplasm. Further investigations determined that the calcitonin gene *CALCA* encodes two alternative mRNA species, calcitonin and calcitonin gene–related peptide (CGRP), and that this alternative splicing was responsible for the decline in calcitonin expression in cell lines that switched to CGRP synthesis instead. Under normal physiological conditions, the processing of the *CALCA* gene transcript is tissue specific. In the thyroid C cells, mRNA transcribed from the calcitonin gene encodes a precursor of the hormone calcitonin, whereas in neuronal tissue, alternative splicing of the RNA generates mRNA encoding the precursor of the neuropeptide CGRP (181). A second, separate gene that encodes CGRP was identified later in both humans and rodents (8, 99). The product of this gene was named βCGRP, with the previously discovered *CALCA*-encoded product becoming αCGRP. βCGRP is the only mature transcript of the *CALCB* gene.

An additional product of the *CALCA* gene is procalcitonin, a 116–amino acid precursor of calcitonin. Under normal physiological conditions, the expression and processing of *CALCA* mRNA into the form that encodes procalcitonin is mostly restricted to C cells of the thyroid, where the precursor protein is quickly cleaved to generate mature calcitonin. The concentration of procalcitonin in the circulation in these conditions is very low (63). In the presence of a bacterial infection, the expression of the *CALCA* gene is induced in many organs and tissues, producing a rapid and substantial increase in the circulating levels of procalcitonin. Studies have shown that procalcitonin production is stimulated directly by bacterial endotoxins and lipopolysaccharides and indirectly by inflammatory mediators. However, the function of procalcitonin synthesized under microbial infection is still unclear. The rapid increase of procalcitonin in the circulation is specific to bacterial infection and is not seen during viral infection or inflammation of other causes. There-

fore, procalcitonin is currently in clinical use as a biomarker for assessing risk of sepsis and septic shock (208). Procalcitonin has also been examined as a biomarker for bone and joint infection, and a meta-analysis of studies including a total of 583 patients concluded that it may be a useful predictor of osteomyelitis or septic arthritis (188). Recently, procalcitonin has been shown to inhibit osteoclast differentiation in bone marrow cultures, and interestingly, this activity was not mediated by the calcitonin receptor (CTR) (119).

3. Amylin

One of the pathophysiological features of type 2 diabetes is the presence of amyloid in the pancreatic islets of Langerhans. Amylin, or islet amyloid polypeptide (IAPP), was purified from pancreatic deposits of patients with type 2 diabetes (40) and from human insulinoma (217). Amylin is a 37–amino acid peptide that is structurally similar to CGRP, with ~50% identity between the two peptides. Human amylin monomers are soluble but can aggregate to form amyloid in type 2 diabetes patients and also spontaneously, in a concentration-dependent manner in vitro, in which they form amylin fibrils (124). The role of the amylin aggregates in the development of β cell lesions in type 2 diabetes is not entirely clear yet, although there is evidence that they contribute to cell death and the loss of islet β cell mass. Recent results from transplantation of human and transgenic animal islets suggest that oligomers of amylin play a key role in the progressive failure of β cells (25, 216). Shortly after the purification of amylin from the amyloid deposits, amylin was also found to be present in β cells of the healthy pancreas. Amylin is stored in the same cellular granules that contain insulin, although the concentration of amylin in the granules is only ~1%–2% of that of insulin. Amylin is cosecreted with insulin; hyperglycemia stimulates amylin secretion, whereas hypoglycemia reduces it (6, 164). The effect of amylin on glucose metabolism is opposite to that of insulin, as it stimulates glycogen breakdown in skeletal muscle (221). Apart from pancreatic β cells, amylin is produced in the gastrointestinal tract and the nervous system (226). In healthy individuals, circulating amylin levels are 5–10 pmol/L, rising to 10–20 pmol/L following a meal (67). Higher levels of circulating amylin were found in humans and animal models of obesity and type 2 diabetes (24, 88, 151, 184).

4. Adrenomedullin

Adrenomedullin was discovered by Kitamura et al., who isolated the peptide hormone from human pheochromocytoma by screening for factors that elevate cAMP levels in platelets (121). The same study showed that the peptide is also highly expressed in normal adrenal medulla and that it elicits a potent and long-lasting hypotensive effect, suggesting that adrenomedullin was a newly identified hormone that regulates blood pressure. Further studies demonstrated high expression levels of adrenomedullin throughout the vasculature and in cardiovascular organs, an expression pattern that was considered to reflect its central role as a vasodilator (28, 145). Other groups found that adrenomedullin is expressed in many tissues, and marked elevation of circulating adrenomedullin was found to be triggered by essential hypertension, renal failure, sepsis, and normal pregnancy (28). Adrenomedullin regulates the proliferation, migration, and differentiation of various cell types and affects a large number of physiological and pathological processes, including hormone release, inflammation, and oxidative stress (189).

5. Intermedin (adrenomedullin 2)

In fish, five different genes (*Adm1–5*) that encode a family of five adrenomedullin peptides have been identified, whereas in mammals, only one additional adrenomedullin family member has been found (195). In 2004, two groups discovered the mammalian gene *ADM2*, which encodes a peptide with high similarity to adrenomedullin, and was named intermedin or adrenomedullin 2 (177, 195). Takei et al. identified intermedin in human, mouse, and rat and determined that, in mice, intermedin mRNA was expressed in the submaxillary gland, kidney, stomach, ovary, lymphoid tissues, and pancreas, but not in the adrenal medulla. Intravenous injection of intermedin in mice decreased arterial pressure more potently than adrenomedullin. Roh et al. found that, in the rat, intermedin is primarily expressed in the pituitary and gastrointestinal tract and showed that intraperitoneal administration of intermedin decreased blood pressure in both normal and spontaneously hypertensive rats (98, 177).

6. CTR-stimulating peptide

CTR-stimulating peptide (CRSP) was isolated from porcine brain (114). The isolation of two additional similar peptides from porcine established a family of CRSP-1–3, with high degree of similarity to CGRP (115). CRSP-1 is expressed mainly in the thyroid gland and the central nervous system. Administration of CRSP into anesthetized rats decreased serum calcium, but did not alter their blood pressure. CRSPs have been identified in other mammals, including cattle, dog, and horse, but are absent in human, rat, and mouse (115).

B. Synthesis and Structure

Peptides of the calcitonin family in human are encoded by five genes with high degree of homology, suggesting that the family was derived from a primordial gene through duplication and mutation. Three of the genes, *CALCA, CALCB*, and *ADM*, are located on chromosome 11, whereas *IAPP* and *ADM2* are on chromosome 12 and 22, respectively. *CALCA* spans ~5.6 kb and contains six exons; exon IV encodes mature calcitonin and exon V encodes mature αCGRP, whereas exons I–III are included in both calcitonin and αCGRP mRNA (22). The splicing of the *CALCA* gene

is tissue specific; in thyroid C cells, 99% of the primary RNA transcript is processed to produce calcitonin mRNA, whereas in neuronal tissue 95% is processed to encode αCGRP mRNA (137). The *CALCB* gene has similar structure to *CALCA*; however, because of sequence variation, the only mature peptide encoded by this gene is βCGRP, which differs from αCGRP by one amino acid in the rat and three amino acids in human. *ADM* encodes adrenomedullin and includes four exons, with exon IV encoding for the mature adrenomedullin peptide. The *IAPP* gene that encodes amylin and the *ADM2* gene that encodes adrenomedullin 2 contain three exons each, with exon III encoding the mature peptide.

All the calcitonin-family peptides are synthesized as precursor proteins that undergo sequential proteolytic processing steps and post-translational modifications to produce the mature peptides. The peptides of the calcitonin family share a number of common structural features: a ring structure formed by a disulfide bridge between two cysteine residues at the NH_2-terminal, an α-helical mid-region, and an amidated amino acid at the carboxyl-terminal **(FIGURE 1)**. The NH_2-terminal and COOH-terminal are highly conserved among calcitonin, CGPR, and amylin, whereas the middle region is more divergent (21). Studies of structure/function have established that the ring structure at the NH_2-terminal of the peptides is essential for receptor activation (17). Thus, linear peptides produced by truncation of the NH_2-terminal domains, including amylin$_{8-37}$, αCGRP$_{8-37}$, and adrenomedullin$_{22-52}$, have been used as antagonists of the parent molecules, as they bind the receptors but fail to activate them (21). In contrast, the NH_2-terminal ring structures (amylin$_{1-8}$, αCGRP$_{1-8}$, and ADM$_{15-22}$) have been shown to retain some of the biological activity of the parent molecules (47, 51).

Calcitonin has been identified in a great number of species. In nonmammalian species, including birds, fish, and reptiles, calcitonin is derived from the ultimobranchial body (70, 162). Interestingly, in all the species examined, the cysteine residues at positions 1 and 7 and the overall length of 32 amino acids are conserved. Calcitonin preparations from two teleost species have been developed and used clinically. Elcatonin (Asu1–7 eel calcitonin analog) is a derivative of eel calcitonin, in which the NH_2-terminal amino group was replaced by a hydrogen atom and the disulfide-bond replaced by ethylene linkage (153). These modifications increased the stability of elcatonin in comparison to the parent molecule while retaining the biological activity. Elcatonin has been trialed for a number of indications and was recently shown to alleviate pain and inhibit bone resorption in patients with osteoporotic vertebral fractures (198). Salmon calcitonin (sCT) differs from eel calcitonin by three amino acids and shares 50% sequence identity with the human peptide. sCT has much higher biological potency in humans than hCT, and has, by far, been the most widely used preparation in clinical practice (33).

C. Receptors

CTR belongs to the seven-transmembrane domain class II (family B) G protein-coupled receptors (GPCRs), a group that includes other receptors that bind regulatory peptides, including PTH/PTH-related peptide, glucagon, vasoactive intestinal polypeptide, and secretin. The first CTR cDNA was cloned from porcine (134), followed by the cloning of the receptors from human and rat (7, 81, 127, 187). CTR is expressed in several tissues, including epithelial kidney cells, the central nervous system, and mature osteoclasts (172). The gene encoding the human CTR, *CALCR*, is located on chromosome 7 and contains 14 exons spanning 150 kb. A number of different isoforms of CTR are produced by alternative splicing; the most common ones differ from each other by the presence or absence of a 16–amino acid sequence in intracellular domain 1 (70). Two isoforms are also found in rodents, with a difference in a 37–amino acid region in extracellular domain 2. The insert negative form is the predominant one in both humans and rodents. The alternative splicing that produces the two isoforms, which appears to be cell type specific, has functional implications, as it affects both the affinity of the receptors to ligands of the calcitonin family and the downstream signaling mechanisms (70).

CTR-like receptor (CRLR) was cloned independently by two groups as a novel receptor with high sequence homology to the CTR (31, 163). The gene encoding CRLR in humans, *CALCRL*, is located on chromosome 2 and contains 15 exons spanning over 103 kb of genomic DNA. For

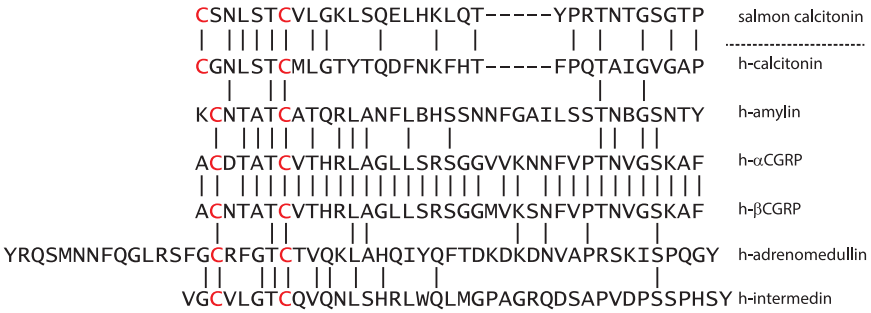

FIGURE 1. Amino acid sequences of peptides of the calcitonin family. Salmon calcitonin and the sequences of peptides of human origin are presented. All peptides are amidated at the carboxy-terminal. Identical amino acids are indicated by vertical lines, and the conserved cysteine residues at the amino-terminal appear in red.

several years, CRLR was an "orphan receptor," with no known ligands (72), until McLatchie et al. (146) discovered the family of receptor-modifying proteins (RAMPs) that form dimers with CRLR to produce specific receptors for the calcitonin-family peptides. Three RAMPs have been identified (RAMP1–3), and although they share only ~30% amino acid sequence identity, all have a similar structure: an extracellular NH_2-terminal, a single transmembrane α-helix, and a short intracellular COOH-terminal. The extracellular domain contains six cysteine residues in RAMP1 and 3, but only four residues in RAMP2. Evidence suggests that disulfide bridges between cysteines 2–4 and 3–6 are essential for CRLR/RAMP complex stability and function (90).

Dimerization of CRLR with RAMP1 forms a specific, high-affinity receptor for CGRP, whereas CRLR dimerization with either RAMP2 or RAMP3 produces receptors with high affinity to adrenomedullin (146). Later studies have shown that CRLR/RAMP complex also functions as receptor for intermedin, which binds in a nonselective manner to either of the three RAMPs/CRLR dimers (177). In addition, RAMPs were found to interact and modulate the pharmacology of CTR (11, 37, 156). Although CTR itself binds calcitonin, complexes of CTR with either of the three RAMPs form specific amylin receptors. Thus, the combinations of the CTR, CRLR, and RAMP1–3 have been recognized as the receptors for all the peptides of the calcitonin family, and a consensus nomenclature has been established **(FIGURE 2)** (37, 156, 169).

Pharmacological studies of the interactions between peptides of the calcitonin family and their receptors have shown that each of the receptor combinations typically binds one of the calcitonin-family peptides with high affinity, whereas other members of the family can bind to the same receptor dimer with lower affinities (21). The specific binding affinities are also dependent on the experimental system (155). The cross-reactivity of members of the calcitonin family with the various receptor combinations presents a challenge for interpretation of experimental results, as deficiency in one specific component can be masked by interactions of the remaining members of the peptide and receptor families.

The regulation of GPCRs is a very active field of research, as GPCRs are the most commonly used drug targets, and it is estimated that 30%–50% of all medications currently in clinical use act through ~80 members of this receptor family. Pharmacological and molecular studies of the RAMP family uncovered novel mechanisms of regulation of GPCRs (89, 123). RAMPs have been found to regulate the activity of CTR and CRLR through a number of mechanisms: *1*) RAMPs act as pharmacological switches, modifying the binding specificities of the GPCRs to their ligands. The crystal structure of CRLR/RAMP1 and CRLR/RAMP2 has been recently solved, and it was found that RAMPs alter GPCR ligand binding pocket first by allosteric changes and then by providing distinct contact sites for ligand interaction (20). *2*) Receptor trafficking: CRLR by itself does not function as an independent receptor, as it is not expressed

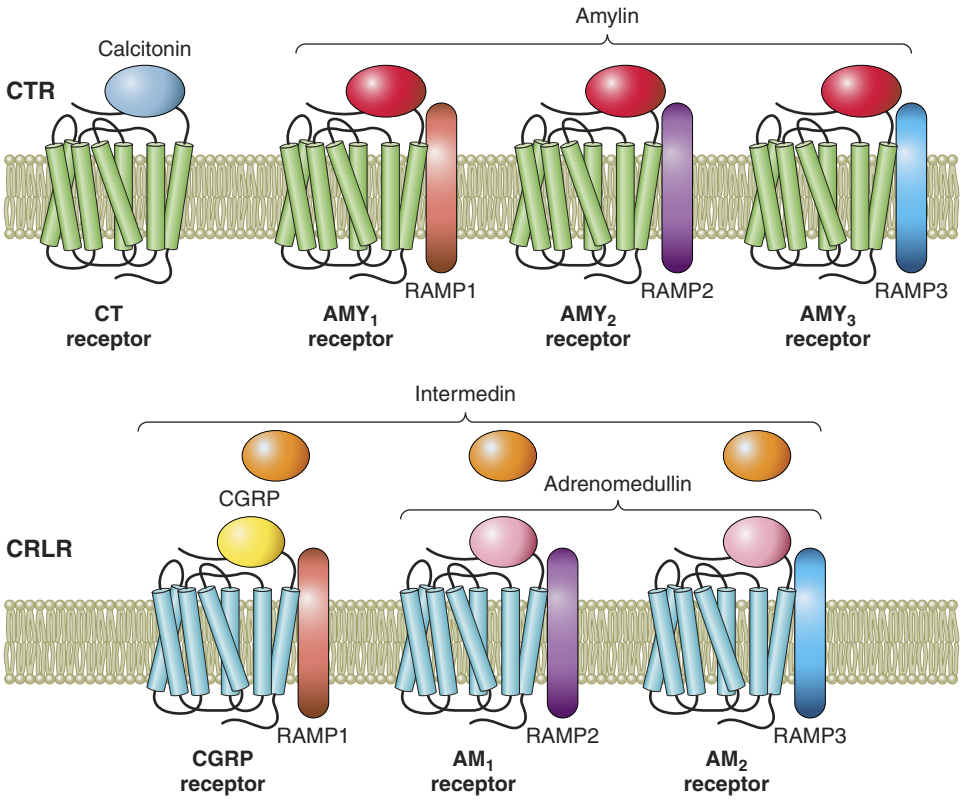

FIGURE 2. Receptors and ligands of the calcitonin family. The seven-transmembrane calcitonin receptor (CTR) by itself binds calcitonin, and when in a dimer with one of the three receptor activity–modifying proteins (RAMPs), it produces a receptor with high affinity to amylin. Calcitonin receptor–like receptor (CRLR) requires a RAMP partner to be presented on the membrane and bind its ligands, forming a high affinity CGRP receptor when associated with RAMP1 and adrenomedullin receptor with RAMP2 or RAMP3. Intermedin binds to dimers of CRLR with any of the RAMPs. The official names of the receptors are indicated below each diagram.

on the cell membrane. RAMPs are necessary to chaperone CRLR to the cell membrane. *3*) Receptor desensitization: following ligand binding, the complex of GPCR and RAMP are typically internalized by the cell. A study of CRLR in complex with the three different RAMPs found that while in complex with RAMP1 and RAMP2, internalization of the receptor is followed by degradative pathways; RAMP3 causes CRLR to be recycled back to the cell surface, allowing for rapid receptor resensitization (19, 123). *4*) Signaling: studies of the amylin receptors (AMY_{1-3}) found that downstream signaling pathways of intracellular calcium production depend on the specific interacting RAMP and suggested that RAMP-complexed receptors have a different signaling profile to that of CTR expressed in the absence of RAMPs (152). A recent study of the pharmacology of CRLR/RAMP receptor complex demonstrated that the receptors display both ligand- and RAMP-dependent signaling bias among the downstream Gα subunit activation (218).

Pharmacological studies of receptor/ligand interactions of the calcitonin family explored the mechanisms that contribute to the greater potency of sCT in comparison to hCT. A recent study demonstrated that these ligands are equipotent during short-term stimulation. However, long-term stimulation with sCT results in sustained activation of CTR, whereas hCT loses its activity much earlier (*9*). Pharmacological studies have established that binding of sCT to rat and human CTRs is essentially irreversible, whereas hCT rapidly dissociates from the receptor. This dissociation was found to be independent of G protein coupling and related to the ability of the ligand to form amphipathic α-helical secondary structure. The higher potential of sCT to form these structures, in comparison to hCT, appears to depend on its NH_2-terminal residues. In structure/function studies, chimeras of sCT and hCT were made, and sCT peptides, which had the NH_2 terminus substituted by the 13–21 residues of hCT, completely dissociated from receptors, whereas combinations of sCT (1–16) and hCT (17–32) retained the irreversible binding of sCT (92). Recently, studies that compared the binding of sCT and hCT to the CTR have found that the different ligands not only affect receptor conformation, but also modulate the bound G protein and the kinetics of downstream signaling (*9*, 75).

II. THE ACTIVITY OF THE CALCITONIN-FAMILY PEPTIDES IN BONE: IN VITRO STUDIES AND IN VIVO STUDIES IN WILD-TYPE ANIMALS

A. The Role of Endogenous Calcitonin in Hypercalcemia

The finding that calcitonin reduces the level of calcium in the circulation led to the hypothesis that its physiological role might be in restoring normal levels of serum calcium in states of hypercalcemia. This hypothesis was tested in a number of early in vivo studies in rats that were either parathyroidectomized (PTX) to remove PTH-secreting cells or thyroparathyroidectomized (TPTX) to remove both PTH- and calcitonin-secreting C cells. When hypercalcemia was induced directly by injection or infusion of calcium, the presence of the thyroid gland was necessary to lower the levels of circulating calcium (78, 196). Similar results were seen when hypercalcemia was induced by injection of parathyroid extract or with partially purified PTH preparation, as serum calcium levels were significantly higher in TPTX rats, confirming the thyroid origin of calcitonin and its role in acute hypercalcemia (94). Secondary hyperparathyroidism and resistance to the calcemic action of PTH are a common finding in renal failure. Rodriguez et al. have shown that the presence of the thyroid gland and the production of endogenous calcitonin are important in decreasing the calcemic response to PTH in rats in both PTH-induced hypercalcemia in the context of renal failure and in diet-induced hyperparathyroidism (176). A physiological role for calcitonin in protection against bone loss, induced by other hormones, has also been suggested. Calcitonin-deficient TPTX rats that were treated with PTH to induce hypercalcemia showed a significant cancellous bone loss in the proximal tibia, whereas calcitonin-sufficient PTX rats had no bone loss, suggesting a protective role for calcitonin in these conditions (225). Following observations that ovariectomy (OVX)-induced osteopenia in rats is associated with a decrease in circulating calcitonin, it has been suggested that calcitonin mediates estrogen deficiency–induced bone loss. However, experimental evidence has not provided support for this hypothesis. For example, the OVX-induced decrease in femur density and calcium content were similar in thyroidectomized rats and in animals with intact thyroid (109).

The main strength of the studies described above is the use of in vivo animal models that allowed the careful manipulation of hormone levels by removal (and in some cases, autotransplantation) of the thyroid, parathyroid, and the ovaries. These studies confirmed that the thyroid is the main source of calcitonin and demonstrated the capacity of endogenous calcitonin to respond to hypercalcemia induced by different protocols. Despite these obvious advances in scientific knowledge, these studies did not provide the answer to the fundamental question of the physiological role of CT, but rather defined its role in a pathological environment. In later years, the development of genetically modified mouse models have provided a much better experimental platform for further investigations of the role of endogenous calcitonin in situations of calcium stress as well as the physiological role of calcitonin.

B. Osteoclasts and Bone Resorption

Mature osteoclasts, formed by fusion of hematopoietic precursor cells, are unique multinuclear cells whose primary

function is to resorb bone. The differentiation and activity of osteoclasts are regulated by a large number of local and systemic factors. Early stages of osteoclast differentiation are initiated by the binding of macrophage colony–stimulating factor (M-CSF) to its receptor c-FMS, which, in turn, induces the expression of receptor activator of nuclear factor kappa–B (RANK), a membrane protein expressed by preosteoclasts. The interaction between RANK and RANK ligand (RANKL), which is expressed by cells of the osteoblast lineage, is the major trigger of osteoclast differentiation and activation. Osteoprotegerin (OPG), a decoy receptor secreted from cells of the osteoblast lineage, binds to RANKL and inhibits the RANK/RANKL interaction in a competitive manner. Thus, the ratio between the levels of OPG and RANKL is a key factor in the control of osteoclast activation and bone resorption (120). Mature osteoclasts degrade bone extracellular matrix through a specialized mechanism, which has been studied in detail. When fully differentiated osteoclasts come into contact with mineralized bone matrix, they form a "sealing zone," an F-actin–rich, ring-like structure that surrounds the enclosed space of the resorption lacuna. The membrane of the osteoclast within the sealing zone is folded and forms a ruffled border through which protons and matrix-degrading enzymes are released in the process of bone resorption.

Studies carried out shortly after the discovery of calcitonin established that inhibition of bone resorption is the main mechanism for the rapid drop in circulating calcium levels induced by calcitonin (230). In the following years, the activity of calcitonin in osteoclasts has been described in detail, and with the discovery of the additional members of the calcitonin family, their activities in osteoclasts have also been investigated and compared with that of calcitonin (53). The expression of CTR, CRLR, and RAMP1–3 has been detected in osteoclasts, suggesting that all the members of the calcitonin family can bind to these cells (84).

1. Calcitonin

Binding of calcitonin to its receptor on osteoclasts has major and immediate consequences: within minutes, there is a loss of the ruffled border, followed by cell retraction and arrest of cell motility and bone resorption (29, 30). A number of signaling mechanisms mediate the activity of calcitonin in osteoclasts; the inhibition of motility and induction of a quiescent state are cAMP dependent, whereas the retraction and disruption of the resorption activity in the sealed zone are mediated through intracellular calcium signaling (3, 229). Studies of the mechanisms involved in the detachment of the sealing zone found that calcitonin modulates the phosphorylation and intracellular distribution of Src and of the tyrosine kinase Pyk2, which is highly expressed in osteoclasts and is localized mainly in the sealing zone (190, 231). A short pretreatment of mature mouse osteoclasts with sCT had no effect on the number of mononuclear or multinuclear osteoclasts that developed in culture but reduced the capacity of the pretreated cells to resorb bone (212). The cells pretreated with sCT produced pits of smaller size than control cells, suggesting a lasting inhibitory effect of sCT on osteoclast motility.

A number of studies investigated the effect of calcitonin on early stages of osteoclast differentiation. Cornish et al. have shown that in murine bone marrow cultures stimulated to generate osteoclasts by $1,25(OH)_2D_3$, calcitonin dose dependently decreased the number of tartrate-resistant acid phosphatase (TRAP)–positive multinucleated cells and substantially reduced the ratio of TRAP-positive multinucleated to mono- and binucleated cells, indicating an inhibitory effect on fusion of osteoclast precursors (44). In contrast, an earlier study of murine bone marrow cultures treated with $1,25(OH)_2D_3$ found that the presence of sCT had no effect on the number of mononucleated and multinucleated TRAP-positive cells (106). In cultures treated with sCT, the cells had very low expression of CTR in comparison to the controls, but the overall level of TRAP expression was unaffected by sCT. When the number of nuclei within the TRAP-positive multinucleated cells were counted, the mean number was lower with sCT treatment, indicating a subtle negative effect of sCT on cell fusion. The main difference between the two studies was the protocol of sCT supplementation. In the latter study by Ikegame et al., a continuous treatment protocol was used with sCT added daily at 0.1 nmol/L. In the study by Cornish et al., sCT, at concentrations ranging from 0.1 pmol/L to 1 nmol/L, was replenished every second day, and it is therefore possible that the cells were exposed to fluctuating levels of sCT. The discrepancies between the results of the two studies suggest tight regulation of osteoclast differentiation in response to sCT, which is likely to be controlled by complex downstream signaling mechanisms.

The use of bone marrow cultures to investigate the effect of sCT on osteoclast differentiation cannot differentiate between direct activity on osteoclast precursors and indirect effect mediated by cells of mesenchymal origin that are present in the bone marrow. The potential direct effect of sCT on osteoclast precursors was studied in two experimental systems: in cells isolated from mouse spleen and in a highly purified preparation of macrophages from mouse bone marrow (83). In cultures of mouse spleen cells, and in bone marrow macrophages (BMM) induced to differentiate by M-CSF and RANKL, sCT inhibited the formation of TRAP-positive multinucleated cells and resorption pits. The cultures contained a large number of TRAP-positive mononucleated cells, indicating the inhibition of cell fusion. Surprisingly, no changes were identified in the levels of expression of a number of genes important for osteoclast progenitor cell differentiation. The inhibitory effect of sCT in this experimental system could be reproduced by activation of cAMP and protein kinase A (PKA) (83).

2. CGRP

Similar to the effects of calcitonin, injection of CGRP into rabbits and rats produced hypocalcemia (179, 202). A large number of studies found that CGRP inhibits osteoclast activity and bone resorption, although CGRP's potency is consistently much lower than that of calcitonin. In cells cultured from mouse bone marrow and induced to differentiate into osteoclasts by M-CSF and RANKL, the area of resorption pits was reduced by CGRP in a concentration of 0.1 nM or higher, whereas higher concentrations of 10 nM CGRP were required for the inhibition of TRAP-positive cell formation, suggesting that CGRP acts with higher potency to inhibit osteoclast activity than osteoclast differentiation (213). In mouse bone marrow cultures treated with $1,25(OH)_2D_3$, CGRP also inhibited the formation of TRAP-positive mono- and binuclear cells and the subsequent fusion of these cells to form multinucleated osteoclasts (44). Reduced formation of osteoclasts was also observed in human bone marrow cultures following treatment with CGRP (2). In this experimental system, CGRP was shown to bind specifically to osteoclast precursors and regulate osteoclast development.

The effect of CGRP on bone resorption has also been assessed in preclinical animal models. OVX is a standard procedure performed in rodents and used as a model for estrogen deficiency–related osteoporosis. When injected into OVX rats, CGRP had an inhibitory effect on the increased indices of bone resorption (207). In this experiment, CGRP was less effective than sCT in suppressing bone resorption, although it was tested at a concentration that was 500 times higher than that of sCT. The reduced magnitude and the low potency suggest that this might be a nonspecific effect of CGRP and not necessarily an activity elicited by binding of CGRP to its own receptor (207). In a local injection model, application of CGRP over the calvaria of adult mice had no significant effect on bone resorption (45, 54).

Recent studies have suggested a role for CGRP in protecting the bone against detrimental effects associated with bone implants. Following joint arthroplasty, aseptic loosening can occur as a result of wear particles that are released from the implant and induce local increase in bone resorption. This process of particle-induced osteolysis is a common cause of early implant failure. The presence of CGRP in the skeleton and nerve fibers adjacent to sites of periprosthetic osteolysis led a number of research groups to test the hypothesis that CGRP has a protective role by inhibiting osteolysis by osteoclasts. The effect of CGRP on the catabolic activity of ultra-high molecular weight polyethylene (UHMWPE) particles was studied in vitro in primary human osteoblasts and in the human osteoblast-like cell line MG-63 (117, 224). In both cell types, UHMWPE particles induced RANKL expression and inhibited the expression of OPG, whereas CGRP reduced UHMWPE-induced RANKL expression, suggesting an indirect inhibition of particle-induced bone resorption.

3. Amylin

Early studies have found that, similar to calcitonin and CGRP, injection of amylin strongly induced hypocalcemia with a potency that was either equal or lower than that of calcitonin (59, 77, 138, 220, 228). In mouse bone marrow cultures that were stimulated to generate osteoclasts by $1,25(OH)_2D_3$, amylin inhibited the formation of TRAP-positive mono- and binuclear cells as well as the fusion of these cells and the formation of multinucleated osteoclast-like cells (44). A later study found that the activity of amylin to inhibit cell fusion required the activation of the extracellular signal–regulated protein kinase 1/2 (ERK1/2) in osteoclast precursors (58).

Amylin also inhibits bone resorption by mature osteoclasts, as determined by the reduced number of resorptive pits per TRAP-positive multinucleated cell in bone marrow cells cultured on bone slices (44). In an organ culture system of neonatal mouse calvariae, in which bone resorption was stimulated by $1,25(OH)_2D_3$, amylin increased cAMP levels and reduced both basal and PTH-stimulated resorption (51, 166). Amylin was also shown to inhibit bone resorption in organ cultures of fetal mouse long bones, in which its potency was similar to that of CGRP, yet 60-fold less than that of hCT (197).

In vivo, daily administration of amylin to adult mice, either locally over the calvariae for five days or systemically for one month, produced a reduction of 60%–70% in indices of bone resorption (45, 48). In a study of the activity of amylin in the context of estrogen deficiency in rats, amylin was injected for 30 days starting 60 days after the OVX surgery. In this experimental model, amylin reduced urinary excretion of deoxypyridinoline and reduced trabecular bone loss, whereas it had no effect on cortical bone indices (101).

4. Adrenomedullin

Despite the presence of adrenomedullin receptors on osteoclasts and the ability of adrenomedullin to induce the synthesis of cAMP in these cells, studies have consistently shown that adrenomedullin does not affect the differentiation and activity of osteoclasts. This has been demonstrated in bone marrow cultures stimulated to generate osteoclasts with $1,25(OH)_2D_3$, or M-CSF and RANKL, and in ex vivo calvarial cultures stimulated with adrenomedullin (43, 46, 82). This is in contrast to all other members of the calcitonin family.

Nevertheless, in certain pathological situations that induce bone loss, adrenomedullin appears to act indirectly to in-

hibit bone resorption through the modulation of the inflammatory environment. In an in vitro model, rheumatoid synovial fibroblasts were treated with the proinflammatory factors IL-1β and TNF-α and then cocultured with peripheral blood mononuclear cells. The treated cells stimulated osteoclast formation, whereas the addition of adrenomedullin in this system modulated RANKL and OPG expression in the rheumatoid synovial fibroblasts and attenuated the increased osteoclast formation (227). In an experimental model of collagen-induced arthritis in mice, adrenomedullin and its truncated form, $ADM_{22–52}$, reduced TNF-α, IL-6, and IL-17 expression in the joints and increased the expression of IL-4 and IL-10. This immune regulation was associated with decreased cartilage degradation and systemic bone loss, suggesting a protective skeletal effect (1).

5. Intermedin

The activity of intermedin in osteoclasts differs from that of adrenomedullin and is similar to that of calcitonin, CGRP, and amylin. In cultures of BMM stimulated with M-CSF and RANKL, intermedin inhibited the formation of multinucleated osteoclasts as well as their ability to resorb bone, as measured by the number of pits formed when cultured on bovine bone slices. Intermedin's activity in this experimental system was mediated by cAMP (82). Recent studies suggested that intermedin might be able to inhibit osteoclast formation indirectly, as MC3T3 osteoblastic cells treated with intermedin had reduced expression of RANKL and M-CSF and increased expression of OPG (175).

6. Comparative studies

A number of studies directly compared the activity of different peptides of the calcitonin family on osteoclast formation and activity. sCT, human amylin, and human CGRP all inhibited osteoclast formation in mouse bone marrow cultures treated by $1,25(OH)_2D_3$ (44). sCT inhibited osteoclast differentiation at concentrations of 0.1 pmol/L and above, whereas CGRP and amylin were much less potent and were active at concentrations of 1 nmol/L and above. sCT, but not CGRP or amylin, reduced the ratio of TRAP-positive multinucleated to mono-/binucleated cells (44). Similar results were found by Granholm et al. (83), who demonstrated that sCT and hCT inhibit osteoclast formation in mouse spleen and mouse BMM, with half maximal inhibition at 1 pmol/L for sCT and 3–4 orders of magnitude higher for hCT (83).

The activity of calcitonin-family peptides on osteoclast formation was also investigated in mouse BMM that were either treated with M-CSF alone to expand the population of osteoclast progenitors or with M-CSF and RANKL to induce further differentiation into TRAP-positive multinucleated, osteoclast-like cells (84). In BMM cells treated with M-CSF, the mRNA and proteins of CRLR and RAMP1–3 were expressed, and treatment of the cells with amylin, CGRP, adrenomedullin, or intermedin induced the production of cAMP (84). However, the addition of RANKL was necessary to induce the expression of CTR, and only when CTR was expressed the cells became responsive to sCT. It is interesting to note that amylin was active in BMM cells treated with M-CSF alone, although CTR was not expressed in these cells, suggesting that, in these cells, amylin signals through a receptor other than CTR/RAMP1–3.

The effects of peptides of the calcitonin family on PTH-stimulated bone resorption were determined by measuring ^{45}Ca release from mouse calvaria in organ cultures (82). Comparison of the inhibitory effects of the peptides on bone resorption clearly demonstrated that sCT had the highest potency, with half-maximal inhibition (IC_{50}) at 3 pmol/L, whereas CGRP and amylin had IC_{50} at 10–30 nmol/L, intermedin at 300 nmol/L, and adrenomedullin was without effect (82).

The effects of calcitonin, CGRP, and amylin on isolated osteoclasts have also been compared through the use of time-lapse video microscopy and image analysis. CGRP and amylin reduced osteoclast motility, but only calcitonin reduced both motility and osteoclast retraction. This study demonstrated that the effect on cell motility is mediated via the cAMP signaling pathway, whereas the effect on retraction appears to be mediated by changes in intracellular calcium. The authors concluded that CGRP and amylin activate only the cAMP pathway, whereas calcitonin also acts on osteoclasts via changes in intracellular calcium and suggest that this may contribute to the greater potency of calcitonin in inhibiting bone resorption (4, 5).

7. The "escape phenomenon"

Early studies have demonstrated that when calcitonin was used clinically to inhibit bone resorption in hypercalcemic cancer patients, the effects of calcitonin were lost after a few days, an effect later termed the "escape phenomenon" (158, 219). In vitro, this effect was first identified in organ cultures, in which, initially, calcitonin inhibited the stimulation of bone resorption by PTH, but after prolonged exposure to calcitonin, its inhibitory effect was lost (215). The desensitization of the cells to calcitonin was found to be the result of a ligand-induced internalization of the CTR, as well as inhibition of de novo synthesis of the receptor, and has been confirmed in both human and murine osteoclast cultures (194, 212). In cultures of mouse bone marrow treated with $1,25(OH)_2D_3$, in which multinucleated TRAP-positive cells were abundant, a brief, one-hour treatment with sCT markedly reduced the expression of CTR mRNA, which remained reduced for the 72 h it was monitored (173). Subsequent studies established that the escape phenomenon is not unique to calcitonin, and can be induced by other members of the calcitonin family. When amylin was

tested in the experimental system of neonatal mouse calvaria, it was also found to produce a transient inhibition of bone resorption by PTH (166). More recently, it has been shown that all members of the calcitonin family downregulated the expression of CTR mRNA, whereas CRLR levels remained unchanged (82). This observation indicates that the downregulation of CTR expression is not limited to peptides that directly bind CTR, but can also be induced, probably through an indirect mechanism, by calcitonin-family peptides that bind CRLR/RAMPs (82).

C. Osteoblasts and Bone Formation

Osteoblasts, the mononuclear bone–forming cells, differentiate from mesenchymal stem cells in the bone marrow. Under a tightly coordinated sequence of molecular signaling, preosteoblasts differentiate into mature osteoblasts that produce and lay down the extracellular bone matrix and subsequently mineralize it. Following a period of active bone formation, the osteoblast can either become embedded within the bone matrix and undergo terminal differentiation into osteocyte, remain on the bone surface in a quiescent state, or die through apoptosis.

1. Calcitonin

A number of studies in vitro and in vivo examined the effect of calcitonin on osteoblast proliferation and bone formation. In one of the earlier studies, calcitonin was found to stimulate osteoblast proliferation and bone formation when administered during the initial phases of bone formation, but when administered after the initiation of bone formation, it had an inhibitory effect (214). Subsequent studies found a direct stimulatory effect of calcitonin on osteoblast proliferation as well as increase in indices of bone formation (68, 209). In contrast, calcitonin had no effect on proliferation of primary rat osteoblasts in culture and did not alter bone formation indices in a local injection in adult mice (45, 47). The understanding of any direct effects of calcitonin in osteoblasts is challenged by the reproducible experimental evidence showing that CTR is not expressed in these cells (160). A possible mechanism for the demonstrated activity of calcitonin in osteoblasts could be nonspecific interactions of calcitonin with receptors other than CTR because of the high peptide concentrations used. In addition, it is possible that the primary osteoblast cultures contained some proportion of other bone cells and an indirect effect in osteoblast was measured. Later studies, using genetically modified animals, produced evidence showing that calcitonin does, in fact, regulate bone formation, but rather than a direct effect in osteoblasts, this activity is mediated via osteoclasts and osteocytes (see below).

2. CGRP

Unlike calcitonin, CGRP appears to have a positive effect on osteoblasts in vitro, but does not affect bone formation in vivo. Specific binding of CGRP to cells of rat calvaria was demonstrated (186). CGRP treatment increased cAMP levels in the UMR 106-01 rat osteosarcoma cell line and in other osteoblastic cell lines and primary osteoblast cultures (18, 148, 200). In primary bone cell cultures from neonatal chicken, rat, and mouse calvariae, CGRP increased cAMP levels, whereas calcitonin had no effect on these cells (149). Intracellular calcium is another second messenger involved in CGRP signaling in osteoblast-like cells, as CGRP treatment increased intracellular calcium levels in UMR 106-01 osteosarcoma cells and in two human osteoblastic cell lines: MG-63 and OHS-4 (23, 65, 118). CGRP induced the proliferation of primary osteoblasts, but its potency was much lower than that of amylin (49). Furthermore, the proliferative effect of CGRP was not inhibited by its antagonist $CGRP_{8-37}$ (210). This has raised the possibility that the effects of CGRP and amylin on osteoblast proliferation are mediated by a common receptor, which has a higher affinity for amylin (49). αCGRP was also shown to stimulate osteoblast differentiation in cultured rat bone marrow cells, whereas in this experimental system, βCGRP had no osteogenic effect (96).

In primary cultures of human osteoblast-like cells, CGRP has been shown to inhibit apoptosis through a Wnt/β-catenin–dependent pathway and stimulate differentiation by increasing BMP-2 expression (154, 201). There is also evidence that CGRP induces differentiation of osteoblast precursors (157, 200). Recent studies demonstrated that CGRP induced the differentiation of bone marrow stromal cells into mineralizing osteoblasts in cells derived from either healthy or OVX rats (133, 213). The effect of CGRP on differentiation of bone marrow stromal cells into osteoblasts appears to be mediated via induction of the Wnt/β-catenin pathway (232). Studies of osteoblast differentiation in three-dimensional cultures have also demonstrated that CGRP promotes osteogenesis of primary rat osteoblast-like cells cultured on Bio-Oss bone graft substitute and the differentiation of adipose-derived stem cells cultured within calcium alginate gels (102, 132). Despite the abundance of evidence that CGRP has positive effects on osteoblasts in vitro, local injection of CGRP in adult mice had no effect on osteoblast indices (45), whereas OVX rats treated with CGRP had no change in bone formation rates (207).

3. Amylin

Early studies have shown that amylin stimulates cAMP production in a pre-osteoblastic cell line and in both primary fetal rat osteoblasts and primary human osteoblasts, thus identifying bone as a potential target of amylin (45, 197, 211). In studies exploring the signaling pathways responsible for amylin's proliferative effect, it was demonstrated that phosphorylation of ERK1/2, most likely through activation of G_i proteins, was required for the mitogenic effect of amylin in rat osteoblast-like cells, as amylin's prolifera-

tive effect was inhibited by PD-98059, a specific inhibitor of this pathway (52). Surprisingly, the proliferative effect of amylin in osteoblasts required the presence of an IGF-1 receptor despite the fact there was no direct binding of amylin to IGF-1 receptor, nor was there a paracrine effect of osteoblast-derived IGF-1 (52).

Structure/function studies of amylin found that the NH_2-terminal octapeptide fragment amylin$_{1-8}$ stimulated primary rat osteoblast proliferation and thymidine incorporation in ex vivo cultures of neonatal mouse calvariae (50, 51). The availability of a short peptide that retains the beneficial bone effects, but is devoid of amylin's activity on energy and carbohydrate metabolism, provides an opportunity for the development of amylin-based therapy for bone. Stable analogs of amylin$_{1-8}$ for potential use in osteoporosis are under development (125, 126).

In vivo, amylin acts to stimulate bone formation. When administered locally over mouse calvariae daily for 5 days, amylin induced two- to fourfold increases in histomorphometric indices of osteoblast activity (45). Systemic administration of amylin to adult mice also produced a 30%–100% increase in these indices (48), whereas in healthy rats, and in osteopenic OVX rats, amylin injections induced an increase of serum osteocalcin (100, 178). Amylin infusion has also produced osteogenic effects in diabetic rat models, although its efficacy varied depending on the model (86). Local injection of amylin$_{1-8}$ over the calvariae of healthy female mice had a positive effect on bone formation, which was greater than that of an equimolar dose of human PTH$_{1-34}$, whereas systemic administration to adult male mice produced a near twofold increase in histomorphometric indices of osteoblast activity (47). In contrast, amylin$_{1-8}$ administration to OVX rats had no effect on parameters of bone formation (66).

4. Adrenomedullin

Similar to amylin, adrenomedullin induced the proliferation of primary human and rat osteoblasts in vitro and in an ex vivo neonatal mouse calvaria culture (46, 87). Adrenomedullin is quite highly expressed in osteoblasts, and therefore could be acting in these cells through a paracrine/autocrine mechanism (159). Unlike other members of the CT family, adrenomedullin has only modest effects on cAMP concentrations in osteoblasts, with studies indicating that adrenomedullin activates ERK1/2 and voltage-dependent calcium channels in these cells (199, 206). Although adrenomedullin does not bind directly to IGF-1R, similar to amylin, its proliferative effect in osteoblasts appears to depend on the presence of IGF-1R (52). Adrenomedullin acts as a survival factor and inhibits osteoblast apoptosis, likely through the ERK1/2 signaling pathway, CREB activation, and the Wnt signaling pathway (130, 206).

When injected locally over the calvaria of adult male mice for five days, adrenomedullin increased indices of osteoblastic activity two- to fourfold and significantly increased bone area (46). Furthermore, systemic treatment with the ADM$_{27-52}$ fragment increased trabecular bone volume and cortical width in the tibia as well as enhancing bone strength in the humerus (43).

5. Intermedin

Intermedin is the least-studied peptide of the CT family in regard to its effects on osteoblasts and bone formation. In vitro studies have demonstrated that intermedin does not affect MC3T3 osteoblast-like cell proliferation or differentiation, yet it does appear to inhibit dexamethasone and serum starvation–induced apoptosis, suggesting an overall positive effect in these cells (175). Further studies of the effect of intermedin in osteoblasts are required to better understand its activity in these cells and its potential effect on bone formation.

D. Osteocytes

Osteocytes are terminally differentiated osteoblasts that reside in lacunae within the mineralized bone tissue and communicate with each other and with other cells and tissues through dendritic processes across a network of interconnected canaliculi. Osteocytes play central roles in the regulation of bone turnover and mineral metabolism. Recent studies found that osteocytes develop osteoclast-like properties and are able to remove bone matrix in their immediate surroundings in a process of osteolysis (171). Osteocytes were found to express genes that had been previously considered as osteoclastic markers, including TRAP and cathepsin K (171). One of the best-studied osteocyte-derived factors is sclerostin, a glycoprotein encoded by the *SOST* gene. Inactivating mutations in *SOST* were identified as the cause of rare diseases characterized by bone overgrowth and general osteosclerosis (10). Sclerostin binds to LRP4 chaperone and LRP5/6 coreceptors and inhibits the Wnt/β-catenin signaling pathway (64). Studies in transgenic animals found that sclerostin potently inhibits bone formation and stimulates bone resorption through a combination of direct effects on osteoclast precursors and indirect effects through the RANKL/OPG pathway. Currently, a humanized monoclonal antibody–targeting sclerostin is in advanced stages of development as an antiosteoporotic drug (55, 182).

The MLO/Y4 cell line was established from bones of transgenic mice expressing the SV40 large T antigen under the control of the osteocalcin promoter (116). MLO/Y4 cells produce extensive dendritic processes when cultured and present other osteocyte-like properties and have, therefore, been used by many research teams as a model for the study of osteocytes. The activity of calcitonin in MLO/Y4 cells

has been studied by a number of groups. Expression of the CTR in MLO/Y4 cells was suggested by the specific binding of [^{125}I]sCT to these cells and the downstream induction of phosphorylation of ERK1/2 and increase in the intracellular levels of cAMP (167). Induction of apoptosis in MLO/Y4 cells by etoposide, TNF-α, or glucocorticoids was prevented by pretreatment of the cells with 5–10 ng/mL sCT. The authors suggest that the therapeutic effect of calcitonin in diseases such as glucocorticoid-induced osteoporosis may be partly due to inhibition of osteocyte apoptosis (167). Later studies demonstrated that the activity of calcitonin in osteocytes is not restricted to the MLO/Y4 cell line. In an animal study that examined the importance of osteoclast activity to the anabolic effect of PTH, young rats receiving anabolic PTH regimen were injected with sCT to transiently inhibit osteoclast activity (80). Unexpectedly, the study found that sCT induced the expression of *Sost* and inhibited the expression of other osteocyte genes: *Mepe* and *Dmp1*. A possible direct effect of calcitonin in osteocytes was suggested and supported by the finding that CTR was expressed in osteocytes that were freshly isolated from rat calvaria, although it was undetectable in cultured primary osteocytes (80). Extension of the study to older animals found that in 6-mo-old rats, sCT had no effect on the expression of *Sost, Dmp1*, and *Mepe* (79). *Calcr* mRNA was expressed in osteocytes isolated from 3-wk-old mice, but its level declined as mice aged and was undetectable at 49 wk of age. The authors concluded that the activity of calcitonin in osteocytes is likely to be physiologically relevant in young rodents (79). Expression of the CTR was also demonstrated in osteocytes isolated from young DMP1/green fluorescence protein transgenic mice (36). Using specific lineage surface markers to isolate a highly purified preparation of osteocytes, the study confirmed that *Calcr*, *Ctsk*, and *Acp5* (TRAP), all considered as osteoclast-marker genes, are, in fact, also expressed in osteocytes (36).

E. Interpretation of In Vitro and In Vivo Studies in Wild-Type Animals

FIGURE 3 presents a summary diagram of the activity of the calcitonin-family peptides in bone cells in the context of bone remodeling. Although the activities of the peptides vary in both specificity and potency, it is evident that an overall positive effect is produced by the calcitonin-family peptides in bone. It is important to note that the experiments discussed in the current section under the title "The activity of the calcitonin-family peptides in bone in vitro studies and in vivo studies in wild-type animals" were conducted using a vast range of peptide concentrations. Under normal physiological conditions, serum levels of the calcitonin-family peptides in humans are within the picomole per liter range. In the studies described above, peptides were used from this low physiological concentration and up to concentrations as high as micromole per liter. With the exception of CGRP, which can reach high local concentrations in the bone microenvironment, experiments testing peptides of the calcitonin family at high pharmacological concentrations have to be considered as such and are not necessarily directly relevant to the physiological activities of these peptides. Furthermore, the fact that sCT is widely used in experimental systems of mouse, rat, and human certainly restricts the interpretation of the results to pharmacological, rather than physiological, conclusions. However, although the physiological relevance might be questionable, the study of sCT and amylin at high concentrations is clinically relevant given the use of sCT as a drug for different indications and the current use of the synthetic analog of human amylin, pramlintide, in patients with diabetes (170). As discussed below, the development of genetically modified animal models was critical in advancing our understanding of the physiological bone activity of the calcitonin-family peptides.

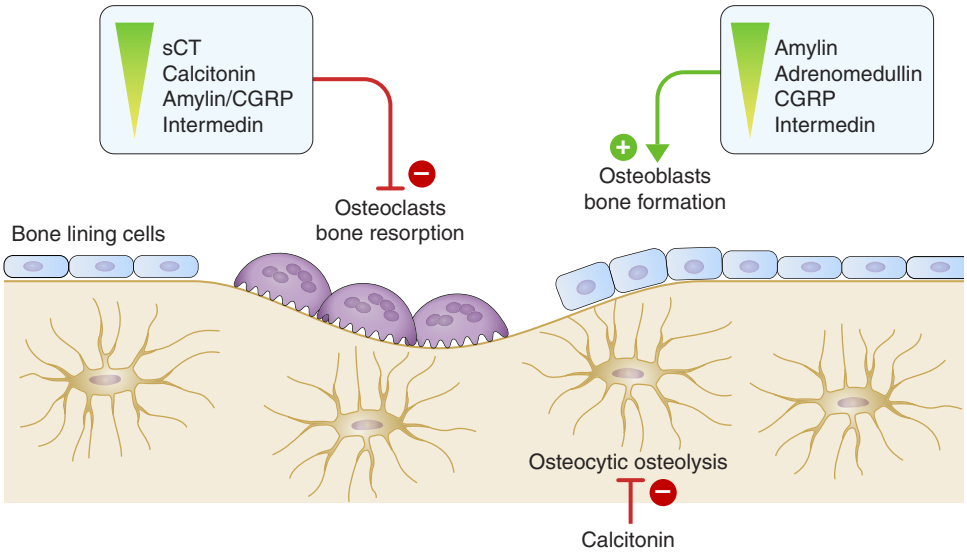

FIGURE 3. The effects of calcitonin-family peptides on bone cells. In vitro studies have established an overall positive effect of the calcitonin-family peptides in bone. Calcitonin, CGRP, and amylin are inhibitory to osteoclasts, and CGRP and amylin also promote osteoblast formation and activity. The osteocyte appears to be a target cell for calcitonin, as studies in genetically modified mice suggested a protective role for calcitonin in osteocytic osteolysis. The peptides are listed in order of potency, which is also indicated by the color gradient. sCT, salmon calcitonin; CGRP, calcitonin gene–related peptide.

III. THE PHYSIOLOGICAL ROLE OF CALCITONIN PEPTIDES IN BONE: STUDIES OF GENETICALLY MODIFIED ANIMALS

A. Calcitonin: Physiological Role

Since the discovery of calcitonin over 50 yr ago (42), its pharmacological activity to lower circulating calcium levels through the inhibition of bone resorption has been thoroughly investigated and described in great detail. It is, therefore, surprising that the physiological role of calcitonin is still unclear. Calcium metabolism and bone mineral density are unaffected in patients with medullary thyroid carcinoma who have long-term excess of endogenous calcitonin levels, or in patients who had undergone thyroidectomy and have undetectable circulating calcitonin (105, 223). Therefore, as deficiency or excess of calcitonin secretion do not appear to have any pathological outcomes, it has been suggested that calcitonin has no physiological role in mammals. One of the codiscoverers of calcitonin expressed the view that calcitonin might be in the process of becoming vestigial, as it appears to be important for survival in fish, but it has no physiological effect on calcium homeostasis or bone metabolism in mammals (93). Similar views questioning the physiological function of calcitonin in mammals were subsequently published under the following titles: "Calcitonin guardian of the mammalian skeleton or is it just a fish story?" (150) and "Calcitonin: physiology or fantasy?" (60). Notwithstanding these skeptical views, the current consensus is that calcitonin has an important role in protection of the skeleton under conditions of calcium stress.

B. Genetically Modified Mice: Calcitonin, CGRP, and CTR

Laboratory investigations in vitro and in vivo established that calcitonin strongly inhibits bone resorption, and therefore, calcitonin-deficient mice were expected to have reduced bone mass in comparison to wild-type (WT) controls as a result of increased bone resorption. However, the bone phenotype of calcitonin-deficient mice proved to be entirely different than expected. In 2002, Hoff et al. generated a mouse strain deficient of the coding sequences of both calcitonin and αCGRP [*Calca* knockout (KO) or CT/CGRP$^{-/-}$] (97). The mice had no developmental defects and had normal baseline-circulating calcium levels. Surprisingly, the *Calca* KO mice had significantly greater trabecular bone volume resulting from increased bone formation. *Calca* KO mice were less sensitive to OVX, maintaining their bone mass, whereas WT mice lost one-third of bone mass over 2 mo after OVX. The phenotype of the *Calca* KO mice suggested that the osteoblast is the primary target of calcitonin/CGRP in bone and that calcitonin/CGRP inhibit bone formation. However, as the mice were deficient in both calcitonin and αCGRP, it was unclear which of the two peptides was responsible for the phenotype (97). Shortly after the original publication, the phenotype of αCGRP KO mice that had intact calcitonin was described by the same group (185). αCGRP KO mice had normal levels of circulating calcitonin, and unlike the *Calca* KO, they had reduced bone formation rate and developed osteopenia. Thus, the high bone mass phenotype of the *Calca* KO mice was a result of calcitonin deficiency, whereas αCGRP appears to increase bone formation. A subsequent long-term study of *Calca* KO mice showed that the bone phenotype developed with age. In animals 12 and 18 mo of age, the increased bone formation was accompanied by a parallel increase in bone resorption, with an overall accelerated bone turnover rate that produced hyperostotic lesions in 20% of the *Calca* KO mice. Therefore, the bone phenotype of the older KO mice suggested a physiological role for calcitonin as an inhibitor of bone remodeling (104). In contrast to the age-dependent progression of the phenotype in *Calca* KO mice, αCGRP KO showed a stable phenotype of osteopenia at all the ages analyzed (104). The analysis of the phenotype of the *Calca* KO and αCGRP KO mice is confounded by the presence of an intact *Calcb* gene that encodes βCGRP. However, a study of the bone phenotype of *Calcb* KO mice found no differences between these mice and control WT mice and led the authors to conclude that βCGRP does not have an important role in regulating bone remodeling (103).

Mechanical loading of the ulna can be used as a model for the study of bone adaptive response to mechanical loading. Sample et al. (183) used this model to test the hypothesis that CGRP, released from sensory fibers in bone, contributes to the adaptive response to load. Mechanically induced activation of periosteal mineralization was seen in WT mice but not in αCGRP KO mice. Interestingly, the phenotype of βCGRP KO mice was similar to that of the WT (183).

Transgenic mice overexpressing CGRP were produced and their bone phenotype determined (15). CGRP was introduced under the osteocalcin promoter to direct the overexpression of the peptide specifically to osteoblasts. Increased bone formation rate as well as higher trabecular bone density and volume were determined in transgenic mice overexpressing CGRP in comparison to WT littermates. These findings suggest a positive effect of CGRP in osteoblasts and are consistent with the study showing reduced bone formation rate and osteopenia in αCGRP KO mice (15, 185).

Further investigations of the physiological role of peptides of the calcitonin family in bone focused on the phenotype of receptor-deficient mice. As global deletion of CTR proved to be embryonic lethal, alternative KO animal models were developed to study the physiological effect of CTR deficiency. In hemizygote CTR KO mice (*Calcr*$^{+/-}$), the expres-

sion of CTR in osteoclasts was half of that found in the WT, and thus, these animals were used as a model of *Calcr* haploinsufficiency (58). Similar to the phenotype described for the *Calca*-deficient mice (97), *Calcr*$^{+/-}$ mice had high bone mass caused by increased bone formation without changes in bone resorption (58). These results provide further evidence for the physiological role of calcitonin as an inhibitor of bone formation and suggest that its activity is mediated via CTR. A second animal model was generated using the Cre/loxP system. Unlike the global CTR KO mice, this strain, which had an incomplete deletion of the *Calcr* gene (with some residual level of expression), was viable (62). In this animal model, only male mice had a small increase in bone formation rate, indicating that CTR plays a minor role in the physiological regulation of bone and calcium homeostasis in the basal state. Recently, using a modified technique to knockout the expression of CTR, Keller et al. managed to produce a new strain of viable global *Calcr* KO mice and used it to investigate the underlying mechanisms of CTR bone activity (119). Similar to the previous models of CTR deficiency, the *Calcr* KO mice had high bone mass because of increased bone formation, a phenotype that was then reproduced in mice that had the gene deletion restricted to the osteoclast lineage. The study found that the loss of CTR in osteoclasts increased the levels of sphingolipid transporter 2 (SPNS2), an exporter protein required for the secretion of sphingosine-1-phosphate (S1P), which is a potent inducer of bone formation. Therefore, according to this study, calcitonin binding to CTR on osteoclasts inhibits the expression of SPNS2, causing a drop in S1P secretion and inhibition of osteoblast activity caused by the reduced levels of S1P (119).

C. Calcitonin, CGRP, and CTR: Conclusions and Remaining Uncertainties

The studies of genetically modified animals described above contributed greatly to the understanding of the significance of calcitonin and its receptor to skeletal biology. However, although some conclusions can be drawn from the integration of these studies, other questions remain unanswered and clearly warrant further investigations. In general, both *Calca* KO mice and *Calcr* KO mice were consistently found to have high bone mass because of increased bone formation, although *Calcr* is not expressed in osteoblasts. This apparent contradiction had been resolved by the finding that the skeletal phenotype of an osteoclast-specific *Calcr* KO had increased bone formation, similar to that of the global *Calcr* KO, and that calcitonin increased the levels of osteocyte-secreted sclerostin. Therefore, calcitonin effect in osteoblasts is likely indirect and mediated via the two types of bone cells that express *Clacr*: osteoclasts and osteocytes. Among the questions that require further investigations is the skeletal phenotype of a mouse deficient of calcitonin. So far, the activity of calcitonin has been inferred from the skeletal phenotype of mice deficient of both calcitonin and αCGRP and mice deficient of αCGRP alone; however, a direct investigation of a calcitonin-deficient mouse is missing. Another important problem that had been overlooked is amylin signaling in the *Calcr* KO mouse. Because all the specific amylin receptors known at present include CTR, the analysis and interpretation of the *Calcr* KO phenotype should consider the possibility of detrimental effect on amylin signaling in this mouse model. It is interesting to note that although the pharmacological function of calcitonin in bone cells is to inhibit bone resorption, studies of genetically modified animals determined that calcitonin's physiological role is to inhibit bone formation. A similar discrepancy was identified in studies of the effect of PTH in bone, as PTH is a physiological stimulator of bone resorption that stimulates bone formation in pharmacological use (143).

D. The Role of Calcitonin and CTR in Situations of Calcium Stress

1. Hypercalcemia

The CTR KO mouse model described above (62) was used to study the role of CTR in maintaining calcium homeostasis in hypercalcemia. When hypercalcemia was induced by $1,25(OH)_2D_3$, the peak in serum total calcium was significantly greater in the CTR KO mice, suggesting that CTR is important in situations of calcium stress (62). The same animal model was used to investigate the contribution of osteoclast-expressed CTR to the protective effect against hypercalcemia (204). The study compared the response to hypercalcemia in three mice strains: WT, global CTR KO, and a Cre/loxP mouse model in which CTR was specifically deleted in osteoclasts. The two KO strains had similar responses to hypercalcemia, with peak serum calcium levels 18% higher than the WT because of increased bone resorption. These results suggest that calcitonin protects against hypercalcemia predominantly through inhibition of osteoclast activity.

2. Lactation

Transgenic animal models were used to study the role of calcitonin and CTR during lactation, a physiological state in which the maternal skeleton rapidly demineralizes to supply calcium to the milk. In the *Calca* KO model, deficient in both calcitonin and CGRP, spine bone mineral content dropped during 21 days of lactation by over 50%, whereas in the WT controls, the drop was only of 23.6% (222). Thirteen days after weaning, spine bone mineral content returned to baseline values in the WT mice, whereas in the *Calca* KO mice, it took 18 days to reach these values. To determine whether the effect in this double-KO mouse model was due to the deficiency in calcitonin or in CGRP, groups of mice were treated with either sCT or CGRP to replace the missing peptide. Injection of sCT normalized the

bone parameters, whereas CGRP was without effect, indicating that calcitonin is the peptide that plays an important role in calcium balance and preservation of the maternal skeleton during lactation and its recovery after weaning (222).

A physiological role for calcitonin in protecting the maternal skeleton from excessive resorption during lactation has also been suggested by a study of global CTR KO mice (38). At the end of lactation, measures of bone resorption by osteoclasts were similar between the CTR KO mice and the WT controls. However, gene expression analysis of mRNA extracted from whole tibiae found increased levels of a number of genes, including *Catk* and *Mmp13*, in the CTR KO mice. As increased osteocytic osteolysis had been previously demonstrated in mice during lactation, the authors hypothesized that calcitonin deficiency induced the expression of osteolytic genes in osteocytes. Histomorphometric analysis of the femurs at the end of lactation found that osteocyte lacunar area in CTR KO mice was larger than in WT, suggesting a role for calcitonin in inhibition of osteocytic osteolysis and protection of the maternal skeleton during lactation (38).

E. Genetically Modified Mice: Amylin

The bone phenotype of amylin-deficient mice was investigated by Dacquin et al. (58). Amylin deficiency had no effect on the regulation of food intake, body weight, or glucose metabolism in this experimental model. At the age of 24 wk, both male and female amylin KO mice were osteoporotic with vertebral bone volume over tissue volume (BV/TV) ~50% lower than that of WT and trabecular and cortical bone thickness reduced. The number of osteoblasts was similar between amylin KO and WT mice, and dynamic histomorphometry using calcein double labeling demonstrated a similar bone formation rate. The amylin KO mice had an increased number of osteoclasts and an increase in degradation products of collagen in the urine, suggesting that the osteoporotic phenotype was a result of accelerated bone resorption (58). The study also investigated the role of CTR in mediating the bone effects of amylin. Unlike amylin KO mice, hemizygous $Calcr^{+/-}$ animals had high bone mass, and compound hemizygous $Calcr^{+/-}$Amylin$^{+/-}$ mice displayed a combination of abnormalities identified in each of the individual hemizygous KOs. Therefore, CTR is unlikely to be mediating the amylin bone effects. The receptor that mediates amylin's effects in bone tissue is yet to be identified (58). Further studies of the same strain of amylin KO mice compared the bone phenotype between young and adult male and female mice (61). Amylin KO males had increased trabecular thickness at 4 and 6 wk of age and increased femoral length at 26 wk, whereas female mice were no different from the WT controls (61).

In summary, the effects of amylin on osteoclast and bone resorption were generally similar in the different experimental systems; amylin deficiency produced an osteoporotic phenotype because of increased bone resorption, and amylin was shown to inhibit osteoclast differentiation and activity in vitro and reduce bone resorption when administered into WT animals. On the other hand, although positive effects of amylin on osteoblasts and bone formation were demonstrated in vitro and with administration of the peptide into animals, amylin deficiency did not modify indices of bone formation. Although the reason for this discrepancy is not clear, it could perhaps reflect the difference between the physiological effect of amylin inferred from the skeletal phenotype of the amylin KO mice and the high, pharmacological concentrations of amylin used in vitro. In addition, it is important to note that the bone phenotype of the amylin KO mouse is age- and gender-dependent, and it is further complicated by the observation that the amylin activity in bone is not mediated by CTR, although currently there is no known amylin receptor that does not include CTR. Thus, a number of unresolved questions remain, and a better understanding of the physiological and pharmacological effects of amylin in bone would require further investigations in existing experimental models and the development of novel ones.

F. Genetically Modified Mice: Adrenomedullin, CRLR, and Ramp1–3

Development of mice strains deficient of adrenomedullin, *Calcrl*, and *Ramp1*, *Ramp2*, and *Ramp3* produced interesting and some unexpected results (108, 189). Genetic deficiency in either adrenomedullin, *Calcrl*, or *Ramp2* (27, 57, 74) is embryonic lethal at midgestation because of cardiovascular defects and a hypoproliferative lymphatic vasculature, whereas *Ramp1* KO and *Ramp3* KO are viable (57, 131, 203). These findings demonstrate that despite the structural similarity among the three RAMPS, RAMP1 and 3 are unable to compensate for the loss of RAMP2.

The skeletal effects of adrenomedullin deficiency were determined in a conditional KO mouse model that was produced using a doxycycline-dependent Cre/Lox excision of the adrenomedullin gene (144). For unknown reasons, rather than being entirely absent, the levels of circulating adrenomedullin were only reduced by ~50% following doxycycline treatment. Analysis of femora by histology and micro-CT determined increased bone mass and density in adrenomedullin-deficient mice in comparison to WT controls. These findings were unexpected, as previously in vitro and local injection experiments had shown a direct positive effect of adrenomedullin on bone formation (46, 159, 205). The authors suggested that the increased bone mass and density in the adrenomedullin-deficient mice were caused by an indirect mechanism. Candidates that could be involved in such indirect effects are CGRP and ghrelin, two peptides that promote bone formation and had increased circulating levels in the adrenomedullin-deficient mice. The

results from the mouse model produced in this study are confounded by the fact that the KO mice gained weight rapidly after exposure to doxycycline (144). In the second part of the study, the authors investigated the effect of an inhibitor of adrenomedullin (the small molecule 16311) on bone loss in OVX mice. The molecule had no effect on BV/TV in femora of sham controls but protected the OVX mice from reduction of BV/TV, suggesting a role for adrenomedullin in mediating the OVX-induced bone loss (144).

Hemizygous *Ramp2*$^{+/-}$ mice are viable, although they have severe reproductive defects, fetal growth restriction, enlarged pituitary glands, and a number of additional abnormalities (107). Haploinsufficiency for RAMP2 caused significant skeletal defects, including delayed development and reduced mineral content and mineral density in femora of 18-wk-old females in comparison to WT controls. Tibiae of *Ramp2*$^{+/-}$ mice were significantly longer than those of WT controls. Although *Ramp2*$^{+/-}$ mice had an interesting skeletal phenotype, it is difficult to delineate the molecular mechanisms responsible for the abnormalities because of the complex endocrine phenotype of these mice. The skeletal phenotype of *Ramp1* KO and *Ramp3* KO has not been described in detail yet. A study that may have relevance to bone, and specifically to fracture healing, examined wound healing in *Ramp1* KO mice (128). The study found that wound healing and wound-induced angiogenesis were significantly suppressed in *Ramp1* KO mice in comparison to WT controls. Wound healing was also delayed in chimeric mice produced by transplantation of bone marrow from *Ramp1* KO into WT mice, indicating a crucial role for hematopoietic cells recruited into the wound.

IV. CLINICAL USE OF CALCITONIN PEPTIDES FOR BONE DISEASES

The discovery of calcitonin as a potent inhibitor of bone resorption by osteoclasts identified it as a potential therapeutic for conditions of excessive resorption. Although neither the absence nor pathologically high levels of endogenous calcitonin appear to affect bone mass, pharmacological use of calcitonin was considered a promising option for the inhibition of bone turnover, preservation of bone mass, and fracture prevention (105, 223).

A. sCT Administration

Among the various calcitonin preparations that have been used in clinical practice, sCT is the most widely used, as it is ~50 times more potent than hCT. sCT has been shown to be safe, and although a large proportion of patients treated with sCT develop antibodies against it, it is generally believed that these have minimal clinical impact (85). sCT was first introduced to the market in 1974 and was subsequently approved by the United States Food and Drug Administration for use in the treatment of postmenopausal osteoporosis, hypercalcemia, and Paget's disease (33, 165). sCT was initially commercially available as an injectable formulation for intramuscular or subcutaneous use. Calcitonin injections had benign, but uncomfortable, side effects, and compliance to long-term parenteral therapy was low (174). The development of calcitonin as a nasal spray provided a much more convenient option for patients, and nasal spray has been used in the clinic and in many studies of calcitonin in humans. The main disadvantage of the nasal spray formulation is its low bioavailability, estimated to be only 10%–25% in comparison to parenteral injections (112). The latest stage in the development of sCT as a drug is an oral formulation, which promises to improve both bioavailability and compliance (16). In the oral formulation, sCT is linked to a drug delivery agent that interacts with the peptide weakly and noncovalently, increasing the ability of sCT to cross the gastrointestinal epithelium and providing a partial protection from degradation by enzymes in the upper digestive tract (113).

B. sCT for Osteoporosis and Fracture Prevention

sCT was one of the first drugs used as an antiresorptive therapy for osteoporosis. A number of small studies of intranasal or subcutaneous use of sCT showed increases in bone density, reduced number of vertebral fractures, and an association with reduction of the risk of hip fracture (26, 33, 110, 139), whereas others found no bone effects (12). The Prevent Recurrence of Osteoporotic Fractures study was a large, multicenter, prospective, 5-yr, randomized, placebo-controlled study that determined the effect of sCT nasal spray on the risk of new vertebral fractures in postmenopausal women with osteoporosis (35). The study found that a daily dose of 200 IU significantly reduced the risk of new vertebral fractures in the study population. However, this conclusion was later challenged, mainly because of the high dropout rate (59%) that compromised the analysis and because only the 200 IU/day dose appeared to be effective, but a higher dose was ineffective (56). A later study used a combination of noninvasive MRI technology and iliac crest bone biopsies to determine the effects of intranasal sCT on parameters of trabecular microarchitecture at multiple skeletal sites (34). This 2-yr, placebo-controlled trial of 91 postmenopausal osteoporotic women suggested a therapeutic benefit of sCT in maintaining trabecular microarchitecture at some skeletal sites, but not others. In a more recent large, randomized, double-blind, placebo-controlled study, oral sCT was tested for the treatment of postmenopausal osteoporosis in a total of 4,665 women over 36 mo (91). sCT induced modest increases in vertebral, femur, neck, and hip bone mineral density in the treatment group but had no significant effects on the proportion of patients with new vertebral, hip, or nonvertebral fractures. Because of this lack of efficacy in preventing frac-

tures, the development of the orally formulated calcitonin was terminated (91). sCT was initially approved for the treatment of postmenopausal osteoporosis at a time when the only other pharmaceutical treatment available was hormone therapy. At present, sCT use declined, and it is no longer considered an appropriate treatment option for osteoporosis for two main reasons: *1*) a number of studies suggested an association between sCT use and cancer incidence (165). Although the evidence is not strong, it led to a 2012 ruling by the European Medicines Agency that "the benefits of calcitonin-containing medicines did not outweigh their risks in the treatment of osteoporosis and that they should no longer be used for this condition." There is some controversy around this ruling, as it is regarded as being based on unconvincing evidence (71). *2*) The development of bisphosphonates and other effective drugs provide much better options for osteoporosis treatment (165).

C. Paget's Disease of Bone

Calcitonin was the first inhibitor of osteoclasts used for treatment of Paget's disease of bone, a common condition characterized by localized increases in bone turnover (129). A number of observational studies, carried out in the 1970–1980s, established the efficacy and safety of sCT in Paget's disease. sCT was effective in inhibiting bone turnover, providing pain relief, and improving bone structure, healing osteolytic lesions and supporting the formation of normal lamellar bone (33). However, calcitonin produced only partial and short-lived improvements, as resistance appeared to develop following its repeated use. Similar to osteoporosis treatment, bisphosphonates offer better options for management of Paget's disease. The European Medicines Agency recommends that sCT be used for the shortest possible time, at the minimum effective dose in Paget's disease, in patients who do not respond to alternative treatments.

D. Osteoarthritis

Another potential indication for sCT is osteoarthritis (OA), a degenerative joint disease that involves cartilage, subchondral bone, and many of the surrounding tissues (136). A number of mechanisms have been suggested for the beneficial effect of sCT in OA, including the inhibition of subchondral bone turnover and subsequent periarticular bone degradation, a direct activity in chondrocytes to induce the

Table 1. *The main effects of members of the calcitonin family on bone cells and the skeletal phenotypes of peptide-deficient mice*

Gene	Protein	Main Activity in Bone Cells in Vitro	References	Skeletal Phenotype of Knockout Mice	References
Calca	Calcitonin	Osteoclasts: inhibition of activity and differentiation.	(3, 29, 30, 44, 82, 83, 190)	*Calca* KO (CT/CGRP$^{-/-}$): High bone mass, increased bone formation rate, and trabecular bone volume, no change in indices of bone resorption.	(97)
		Osteoblasts: inconsistent findings.	(45, 68, 209)		
		Osteocytes: inhibition of apoptosis, increased expression of the Wnt-signaling inhibitor sclerostin.	(80, 167)	In older mice (12–18 mo), parallel increase in resorption, overall accelerated bone remodeling rate.	(104)
Calca	αCGRP	Osteoclasts: inhibition of activity and differentiation.	(2, 44, 82, 213)	Reduced bone formation rate, osteopenia.	(185)
		Osteoblasts: stimulation of proliferation and differentiation.	(49, 102, 133, 154, 201, 210, 213, 232)		
Calcb	βCGRP	No effect on osteogenesis in bone marrow cultures.	(96)	No skeletal phenotype.	(103, 183)
Iapp	Amylin	Osteoclasts: inhibition of activity and differentiation.	(4, 44, 51, 58, 82, 166, 197)	Osteoporosis caused by accelerated bone resorption. No effect on bone formation rate.	(58)
		Osteoblasts: stimulation of proliferation and differentiation.	(45, 49, 51, 52, 210, 211)		
Adm	Adrenomedullin	Osteoclasts: no effect.	(82)	Embryonic lethal	(27)
		Osteoblasts: stimulation of proliferation and differentiation.	(46, 52, 87, 130, 159)	Partial-KO model: increased bone mass.	(144)
Adm2	Intermedin	Osteoclasts: inhibition of activity and differentiation.	(82)	NA	
		Osteoblasts: no effect on proliferation or differentiation.	(175)		

CGRP, calcitonin gene–related peptide; KO, knockout; NA, not available.

production and secretion of cartilage extracellular proteins, and providing an analgesic effect (33).

A direct effect of calcitonin on chondrocytes has been demonstrated in a number of studies. In a model of degradation in bovine articular cartilage explants, calcitonin induced cAMP production in chondrocytes and inhibited degradation of type II collagen and the increase in matrix metalloproteinase activity (193). In vivo, calcitonin was shown to attenuate type II collagen degradation and to reduce cartilage erosion, extracellular matrix degradation, and subchondral bone damage in experimental animal models of arthritis (32, 140, 192). In addition, OA induced by destabilization of the medial meniscus produced a fivefold increase in cartilage erosion index, whereas transgenic mice overexpressing sCT had ~60% lower erosion index than WT mice (191). A direct effect of calcitonin in chondrocytes requires the expression of CTR in these cells. However, although some studies demonstrate the expression of CTR in chondrocytes (193), others find no expression (135).

In humans, oral sCT reduced circulating carboxy-terminal collagen cross-links II and improved functional disability in patients, albeit in a small test group, selected for active disease (14, 141). However, recently published results of two phase III–randomized, double-blind, placebo-controlled trials that evaluated the effect of oral sCT on symptomatic knee OA found no significant impact on joint space narrowing in the treatment groups (111).

V. SUMMARY

The calcitonin-family peptides and their receptors form a complex network of interacting molecules. Although the primary physiological roles of the peptides are diverse and they affect a wide range of cell types and tissues, the skeletal phenotype of mice deficient of either calcitonin, amylin, αCGRP, or CTR indicates an important role for the calcitonin family in skeletal physiology. The main effects of the calcitonin-family peptides and the characteristics of the skeletal phenotype of mice deficient of the peptides and receptors are summarized in **TABLES 1** and **2**.

A number of questions regarding the bone activities of the peptides are yet to be resolved. One major challenge is the discrepancy between the pharmacological effects observed in bone cells in vitro and in animals injected with the peptides and the physiological roles inferred from the skeletal phenotype of genetically modified mice and clinical observations in humans. A large part of these discrepancies are undoubtedly due to the inherent differences in physiology and genetics between species. To date, the fundamental target of calcitonin-family peptides in bone appears to be the osteoclast. It is not surprising to see discrepancies between animals and humans, as well as between animals of different ages, as the activity of osteoclasts, along with the rate of bone remodeling, are vastly different. There are also differences in pharmacokinetics between species such that pharmacological responses in mice to certain drug concentrations cannot be easily related to responses in humans. Similarly, response to pharmacological interventions changes depending on age, health, and a number of environmental factors that cannot be translated from the laboratory. Despite this, major progress achieved in recent years, including the discovery of the osteocyte as a target cell for calcitonin and the development of animal models with tissue-specific gene KO, has helped explain some of the apparent contradictory observations. Additional answers will undoubtedly

Table 2. *The main features of the skeletal phenotype of receptor-deficient mice*

Gene	Protein	Skeletal Phenotype of Knockout Mice	References
Calcr	Calcitonin receptor (CTR)	*1*) *Calcr*$^{+/-}$ (hemizygous): high bone mass, increased bone formation.	(58)
		2) Incomplete KO model: increased bone formation.	(62)
		3) Global *Calcr* KO: high bone mass, increased bone formation.	(119)
		4) Osteoclast-specific *Calcr* KO: high bone mass, increased bone formation.	(119)
Calcrl	Calcitonin receptor–like receptor (CRLR)	Embryonic lethal	(57, 74)
Ramp1	Receptor activity–modifying protein (RAMP)1	NA	
Ramp2	RAMP2	Embryonic lethal	(74)
		Ramp2$^{+/-}$ (hemizygous): delayed bone development, reduced mineral content, and mineral density.	(107)
Ramp3	RAMP3	NA	

KO, knockout; NA, not available.

be provided in the future with further development of genetically modified animals and thorough investigations of their phenotype at different ages, in states of calcium stress, and in models of accelerated bone turnover.

The use of calcitonin for the management of conditions of accelerated bone turnover was expected to increase with the development of nasal and oral formulations of sCT. However, with the availability of more effective drugs for long-term inhibition of bone resorption, sCT use has declined, and there are no current trials for its use for skeletal disorders. Despite this, there is still a great interest in the calcitonin family of peptides and their skeletal effects, with current studies designed to clarify the activities and physiological roles of this complex and intriguing family of peptides.

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