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Review Article

Hylonome

Deletions of genes encoding calcitonin/α-CGRP, amylin and calcitonin receptor have given new and unexpected insights into the function of calcitonin receptors and calcitonin receptor-like receptors in bone

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Abstract

It has been suggested that skeletal nerve fibers may play important roles in neuro-osteogenic interactions. This view is partly based upon information obtained from immunohistochemical studies, chemical and surgical denervation experiments and clinical observations in patients with stroke and spinal cord injury, indicating the presence of a network of nerve fibers in the skeleton and that defective signalling in skeletal nerve fibers affects remodelling of bone. This view is also supported by data showing that functional receptors for signalling molecules in skeletal nerve fibers are expressed in bone cells and that activation of these receptors leads to profound effects on bone forming osteoblasts and bone resorbing osteoclasts. Convincing evidence for a role of neuronal signalling in bone metabolism has been provided by gene deletion approaches in which it has been shown that leptin-sensitive and neuropeptide Y-sensitive receptors in hypothalamus are important for bone remodelling in mice. Recently, gene deletion experiments have shown that calcitonin gene-related peptide (CGRP), one of the neuropeptides present in skeletal nerve fibers, is an important physiological regulator of bone formation at the level of osteoblast activity. CGRP belongs to the calcitonin (CT) family of peptides also including CT, amylin and adrenomedullin, as well as the recently described intermedin and calcitonin receptor-stimulating peptide. These peptides utilize two seven transmembrane G protein-coupled receptors – the calcitonin receptor (CTR) and the calcitonin receptor-like receptor (CRLR) – which can dimerize with three different single transmembrane proteins, making up the RAMP family. Associations between RAMPs and either CTR or CRLR give rise to seven distinct, molecularly characterized, receptors for CT, CGRP, amylin and adrenomedullin. Deletions of the genes for ligands in the CT family of peptides and for one of the receptors have revealed unexpected findings that have changed our view on the role of these peptides in bone remodelling. It was anticipated that deletions of the *CT/α-CGRP* and *CTR* genes would lead to bone loss, since CT has been shown to inhibit bone resorption *in vitro* and *in vivo* and has been used to treat patients with excessive bone resorption. Surprisingly, it was found that $CT/\alpha\text{-}CGRP^{-/-}$ and $CTR^{+/-}$ mice have increased bone mass due to increased bone formation. Mice with deletion of the amylin gene, however, exhibited bone loss due to enhanced bone resorption. Selective deletion of the *α-CGRP* gene also leads to bone loss, but due to decreased bone formation. Thus, our understanding of the role of the CT family of peptides has been changed dramatically and much more data have to be gained before we fully understand the roles these peptides have in bone biology.

Keywords: Bone, Neuropeptides, Calcitonin, Amylin, Calcitonin Gene-related Peptide, Adrenomedullin, RAMP

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Introduction

Increasing amount of evidence accumulated during recent years have indicated that remodelling of the skeleton is controlled, not only by systemic hormones and local cytokines, but also by the nervous system[1-3]. Immunocytochemical stud-

ies have shown the presence of sensory and autonomic nerve fibers in the periosteum, in cortical and trabecular bone, as well as in bone marrow. These nerve fibers express a variety of neuronal signalling molecules, including neuropeptides, catecholamines and neurotrophins. Surgical and chemical denervation results in a skeletal phenotype that cannot only be attributed to decreased mechanical loading, indicating that neuronal signalling is important for bone remodeling. Similarly, patients with stroke and spinal cord injuries rapidly develop osteoporosis, which also not only can be explained by decreased loading of the skeleton. The fact that osteoblasts and osteoclasts express receptors for several of the signalling molecules present in skeletal nerve fibers and that these receptors have been shown to be functional, not only in a sense that activation leads to increased intracellular signalling but to a shift in the biological activities of the cells also indicates that neuronal signalling molecules are able to regulate bone cell activities. It has recently been elegantly shown that the central nervous system can exert profound effects on skeletal metabolism. Thus, Karsenty and collaborators have demonstrated that mice with deletions either of the gene encoding leptin, or of the gene encoding leptin receptors, result in increased bone mass in the vertebral bodies[4]. The increased bone mass was due to increased bone formation. Since this group was unable to detect leptin receptors in bone and since intracerebroventricular injection of leptin, to leptin-deficient mice, rescued the bone phenotype, it was concluded that it is the hypothalamic receptors recognizing leptin that are responsible for the skeletal phenotype. In a subsequent paper, the same group showed that the hypothalamic neurons causing the previously well-known anorexigenic effect by leptin are distinct from those causing the anti-osteogenic effect[5]. The authors also showed that the hypothalamic control of bone remodeling was mediated by the sympathetic nerve system and by β-adrenergic receptors on osteoblasts, which upon activation leads to inhibition of bone formation. Interestingly, the leptin-deficient *ob*/*ob* mice also exhibit signs of increased bone resorption due to enhanced sympathetic signalling via β-adrenergic receptors[6]. The concept of leptin being anti-osteogenic in the spine has been challenged by Hamrick and collaborators showing that *ob*/*ob* mice have low bone mass in the limb skeleton[7], due to enhanced endosteal bone formation in association with a decreased number of adipocytes[8]. It has also been elegantly shown that neuropeptide Y (NPY) signalling in the hypothalamus is important for bone remodelling. Herzog and collaborators, working at the Garvan Institute in Sydney, have shown that mice with deletion of the gene encoding NPY type 2 receptors have increased bone mass due to enhanced bone formation[9]. Since these receptors are expressed in a variety of different cells it could not be concluded in which cell types NPY signalling is important for skeletal remodelling. There are indications that NPY receptors may be expressed by osteoblasts, although this has not been very much studied and there are no studies showing that NPY receptors in bone cells are functional. For several reasons, the Garvan group then conditionally knocked out the NPY type 2 receptors in the hypothalamus and these mice showed an identical phenotype compared to those in which the receptors were globally knocked out. Thus, two lines of evidence, one being based upon leptin deficient mice and the other upon NPY type 2 receptor deficiency, show that the hypothalamus is important for bone remodelling in mice. It is not know, yet, how the deficiency of NPY signalling in hypothalamus is mediated to bone cells.

The two neuropeptides which have been most extensively studied in the bone field are vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP), which both have been shown to affect the activities of osteoblasts as well as osteoclasts[1]. Thus, we have shown that osteoblasts and osteoclasts express different sub-types of VIP receptors and that activation of these receptors in osteoblasts leads to increased alkaline phosphatase activity, increased mineralization of bone nodules and enhanced expression of IL-6, but not other cytokines in the IL-6 family of cytokines. VIP also regulates the bone resorbing activity of osteoclasts as well as the formation of osteoclasts[10,11].

CGRP has been shown to enhance bone formation and decrease bone resorption *in vivo* and *in vitro*[12]. This peptide belongs to the calcitonin peptide family, which is made up by calcitonin, CGRP, amylin and adrenomedullin and two recently identified members, intermedin and calcitonin receptor stimulating protein (CRSP)[13]. These peptides function as ligands for a complex family of receptors consisting of the calcitonin receptor (CTR), calcitonin receptor-like receptor (CRLR) and three receptor associated modified proteins (RAMPs). In the present review, the knowledge about the function of these ligands and receptors in bone will be summarized, together with the most interesting and unexpected information that has emerged from the phenotype caused by deletions of some of the ligands and receptors. The data have implications for understanding of the role of the nervous system in skeleton and for bone physiology in general.

The calcitonin peptide family

Calcitonin (CT), being synthesized by the thyroid C-cells, was discovered by Harold Copp more than 40 years ago as an hypocalcemic compound[14]. Today, there are indications that also other cells might produce CT, or CT-like molecules, such as brain and prostate[15], although the biological role of these molecules is unknown. CT is expressed in many species including fish, reptiles, amphibians, mammals and birds and is usually a 32 amino acid peptide with a carboxy terminal proline amide and a disulfide bridge between cysteine residues at position 1 and 7. This amino terminal disulfide-bridged ring is shared by all peptides in the CT family of peptides. The amino acid sequence of CT in the amino terminal loop region is highly conserved within species, but displays less homologies in the rest of the sequence. Interestingly, the potency of CT from different species varies a lot, with salmon calcitonin being the most potent. In most assays, salmon CT is at least 1,000-fold more potent than human

CT. Salmon CT has been used for the treatment of diseases with excessive bone resorption such as Paget's disease and osteoporosis, but due to down-regulation of the CT receptors caused by the ligand, the efficacy of CT treatment is limited and because more effective inhibitors of osteoclast activity, such as bisphosphonates, are at hand today, CT is not the first choice of treatment.

CT is synthesized as 136 amino acid precursor molecules, which is processed by proteolytic cleavage and by amidation of the carboxy terminal proline residue before being secreted. The gene transcript encoding CT also encodes α-CGRP, another peptide in the CT family of peptides. Thus, two important signalling molecules, with different functions, are produced by one and the same gene. Cell specific alternate processing of the CT/α-CGRP transcripts is the mechanism regulating the formation of either CT or α-CGRP in different cell types. α-CGRP, being a 37 amino acid peptide, is widely expressed in nerves in both the central and peripheral nervous system and is a potent vasodilator. CT and α-CGRP have some homologies in the amino terminal moiety, but are almost entirely different in the rest of the molecules.

Another molecule that is almost identical to α-CGRP is synthesized as a product of a gene distinct from CT/α-CGRP gene. Thus, β-CGRP is a 37 amino acid peptide that differs from α-CGRP by just three amino acids in humans. This gene is expressed in nerve fibers in very many different tissues. The physiological role of α-CGRP versus β-CGRP expression is not known.

Amylin was originally isolated from the amyloid deposits in the pancreas of patients with type II diabetes. It is a 37 amino acid expressed in pancreatic β-cells, in which it is released concomitantly with insulin. Due to its effects on glucose transport and gluconeogenesis it has been implicated in type II diabetes. The amino acid sequences of amylin and CGRPs are almost identical in the amino terminal loop, but in the carboxy terminal part amylin displays only approximately 40% of amino acid identity with CGRPs.

Adrenomedullin was initially found in pheochromocytoma cells, but later has been found to be expressed in a variety of tissues and cells, including adrenal medullae, endothelial cells, smooth muscle cells, fibroblasts, macrophages, neurons and glial cells. Immunohistochemical studies have shown that adrenomedullin is expressed in endocrine pancreas, without being co-localized with other known hormones. Interestingly, adrenomedullin has been shown to be expressed also in osteoblasts[16,17]. The fact that adrenomedullin has been found to be expressed by macrophages raises the possibility that it may be expressed by osteoclasts, although this remains to be shown. Adrenomedullin is a 52 amino acid peptide that differs from CT, CGRP and amylin since instead of the amino terminal loop, it has a linear amino terminal extension consisting of 15 amino acids in humans and 13 in the rat. Adrenomedullin is a potent vasodilator, but can bind to many other cells and has also been found to have diuretic and bronchodilatory effects. It has approximately 20% amino acid homology with amylin and CGRP and slightly less with CT.

Intermedin was recently identified using a phylogenetic profiling approach to analyze the GenBankTM/EBI DataBank as a peptide with homology to peptides in the CT family of peptides[18,19]. The putative mature region of intermedin has approximately 28% amino acid sequence homology with adrenomedullin and approximately 20% homology with CGRP. The amino terminal part has, similar to CT/CGRP/amylin, a disulfide-bonded loop. The fact that intermedin can activate CRLR, in the presence of RAMP, further established the relationship to the CT family. Intermedin has been found to be expressed in the gastrointestinal tract and in the pituitary. Intermedin decreases blood pressure and suppresses gastric emptying activity and food intake.

Calcitonin receptor-stimulating peptide (CRSP) is also a recently discovered peptide found to be expressed in the porcine brain and thyroid glands[20-22]. CRSP has 60% amino acid homology with CGRP but has several structural and pharmacological features distinct from CGRP and other members of the CT family. Since the initial discovery of CRSP, two related peptides were identified in a cDNA library from porcine brain, and the CRSPs were designated CRSP-1, CRSP-2 and CRSP-3. All these porcine CRSPs have an amino terminal disulfide-bonded loop and a carboxy terminal amide. However, the three peptides have different affinities to the receptors in the CTR family. Thus, CRSP-1, but not CRSP-2 and CRSP-3, can stimulate cyclic AMP production in CTR expressing cells. Nor can CRSP-2 or CRSP-3 stimulate cyclic AMP formation in cells expressing CGRP, amylin or adrenomedullin receptors. CRSPs have been found in the porcine, bovine and canine species, but not in human or rats. None of the bovine or canine CRSPs can stimulate cyclic AMP in cells expressing the known variants of the receptors for CT, CGRPs, amylin or adrenomedullin. The biological role of CRSPs is not known yet.

Receptors for the calcitonin family of peptides

The **calcitonin receptor** (CTR) belongs to the type II seven transmembrane G protein-coupled receptors to which also the receptors for parathyroid hormone (PTH)/PTHrP, VIP, pituitary adenylate cyclase-activating peptide (PACAP), glucagon, secretin, growth hormone releasing hormone, corticotropin releasing factor and gastric inhibitory polypeptide belong. The porcine CTR was the first to be cloned by Lin et al. (1991)[23]. Shortly afterwards, the CTR was cloned in human and several other species and characterized[24]. CTR is highly expressed in multinucleated osteoclasts[25], but not in the early osteoclast progenitor cells/macrophages[26]. The CTR is induced in these cells by activation of the receptor RANK. Signalling through RANK, a member of the tumor necrosis factor receptor superfamily, results in a unique signalling pathway by which mononucleated osteoclast progenitor cells differentiate to multinucleated osteoclasts[27-29]. Expression of CTR is a late event during osteoclastogenesis and the receptor can be found on the progenitor cells just prior to multinucleation[26].

Several isoforms of CTR have been found in different cells and osteoclasts have been found to express predominantly CTR1a, but also to some extent CTR1b[15]. The functional role of the different isoforms is not known, although activation of some isoforms by CT does not cause increased levels of intracellular calcium and some isoforms are less potent to stimulate cyclic AMP. Activation of CTR by CT rapidly downregulates the receptor, which is likely one mechanism by which inhibition of bone resorption by CT is transient. The mechanism for downregulation is not fully understood, but involves ligand-dependent decrease of CTR mRNA. Although some osteoblastic cell lines may express CTR[30], most osteoblasts and stromal cells from a variety of different species do not exhibit CTR.

Calcitonin receptor-like receptor (CRLR) was initially reported as an orphan receptor belonging to the type II family of G-protein coupled receptors, but has recently been found to be a receptor component recognized by either CGRP or adrenomedullin[31-34]. CRLR and CTR have overall approximately 55% amino acid sequence homology, but in the transmembrane domain the homology is almost 80%. CRLR can function as a receptor for both CGRP and adrenomedullin, the ligand specificity depending on its association with different RAMPs. CRLR mRNA and protein have been detected in rat and mouse calvarial osteoblasts, UMR 106-06 cell line and the non-transformed mouse calvarial osteoblastic cell line MC3T3-E1[16,35,36]. CRLR mRNA also has been found to be expressed in osteoclasts[37].

Receptor activity-modifying proteins (RAMPs) was cloned in 1998 by McLatchie et al.[38] and RAMP1 was found to be a 148 amino acid peptide in the family of type I single transmembrane proteins with a large extracellular amino terminal domain, a single transmembrane spanning domain and a short cytoplasmic domain[31-34]. RAMP2 and RAMP3 were found in a database search for related proteins. One important function of RAMPS is to control the glycosylation of CRLR and thereby its transportation to the cell membrane. RAMPs can heterodimerize with both CRLR and CTR and these dimeric proteins are expressed at the cell surface and make up different receptors for ligands in the CT family of peptides. The three different RAMPs identified so far share a similar basic structure, including four conserved cysteines in the amino terminal part and approximately a 30% amino acid identity. The different RAMPs are more widely expressed than CTR and CRLR and have been found in many cells and tissues, including cells not expressing CTR or CRLR, and recently also found to be expressed by bone cells. Primary rat calvarial osteoblasts and the rat osteosarcoma cell line UMR106-06 express all three RAMPS[16]. Mouse calvarial osteoblasts and MC3T3-E1cells also express all three RAMPs and treatment with dexamethasone resulted in increased expression of RAMP1 and RAMP2, whereas RAMP3 and CRLR were unaffected[35]. Using laser capture microscopy, Nakamura et al.[37] reported that osteoclasts formed in mouse bone marrow and mouse spleen cell cultures stimulated by M-CSF and RANKL expressed, besides CTR mRNA, also mRNA for CRLR and RAMP2, but not for RAMP1 and RAMP3.

Receptor name	Molecular components
$CGRP_1$	CRLR + RAMP1
AM_1	CRLR + RAMP2
AM_2	CRLR + RAMP3
CTR	CTR
AMY_1	CTR + RAMP1
AMY_2	CTR + RAMP2
AMY_3	CTR + RAMP3

Table 1. The nomenclature for the calcitonin peptide family receptors.

Heterodimerization between different RAMPs and either CTR or CRLR determines the ligand specificity for these receptors. The discovery of these dimerizations and how they change ligand specificity has changed the view how G-protein coupled receptors function. At least seven different receptors have been recognized for the peptides in the CT family of peptides[39]. In contrast to CGRP, amylin and adrenomedullin, CT signalling is not dependent on RAMPs, since CT has its full effect in the absence of RAMPs. However, when CTR is associated with the different RAMPs, the receptor complexes bind amylin and generate different amylin receptors. The CGRP receptor is made up by CRLR dimerized with RAMP1, and when CRLR is associated with either RAMP2 or RAMP3 it functions as adrenomedullin receptors (see Table 1).

Receptor component protein (RCP) is a 146 amino acid protein that is expressed in several tissue and cell lines. RCP co-immunoprecipitates with CRLR. The function of RCP is not fully known, but it seems to be important for the coupling of G protein-mediated signalling of CRLR.

Effects by peptides in the calcitonin family on bone

Calcitonin causes a rapid decrease of serum calcium, which is partly due to inhibition of bone resorption and partly to effects on the kidneys. The acute effect on bone resorption is caused by stimulation of CTR on multinucleated osteoclasts leading to contraction, ceased motility and decreased bone resorbing activity[14]. Because of these effects, CT inhibits bone resorption in organ culture and bone resorbing activity of isolated osteoclasts. The effect is, however, transient and involves both receptor internalization and decreased mRNA expression of CTR. If decreased osteoclast formation also may contribute to the inhibitory effect by CT on bone resorption is much less understood. However, Cornish et al.[40] have reported that CT inhibits osteoclast formation in mouse bone marrow cultures stimulated by $1,25(O)_2$-vitamin D_3, an observation which has recently been confirmed in our laboratory. The mechanism

by which CT decreases osteoclast formation is currently not known. CGRP and amylin can also decrease bone resorption in both organ cultures and in isolated osteoclasts[1]. Interestingly, adrenomedullin does not inhibit bone resorption in mouse bone organ cultures[41]. The finding that CGRP inhibits bone resorption indicates that osteoclasts express CRLR and RAMP1, whereas the lack of effect by adrenomedullin suggests that RAMP2 and RAMP3 are not expressed. However, it has recently been reported that osteoclasts in mouse bone marrow cultures express CRLR and RAMP2, but not RAMP1 and RAMP3[37]. Similar to CT, also CGRP and amylin have been found to decrease osteoclast formation *in vitro*[40].

Amylin, CGRP and adrenomedullin have all been shown to stimulate osteoblast proliferation *in vitro* and to enhance bone formation and mechanical strength *in vivo*[16,41-44]. Interestingly, the anabolic response to adrenomedullin *in vivo* and *in vitro* can be obtained also by the adrenomedullin fragment adrenomedullin(27-52), which lacks the effects by adrenomedullin on vasodilation[45]. In contrast, the anabolic effect by amylin can be obtained by fragments lacking the carboxy terminal part. Since CTR was not expressed in cells used for the *in vitro* experiments it seems as if amylin does not use any of the amylin receptors made up by the CTR associated with RAMP1, RAMP2 or RAMP3. Further evidence for the anabolic effect by CGRP is the finding that transgenic mice expressing CGRP under control of the osteocalcin promoter have increased bone volume due to enhanced bone formation[46]. It has also been found that treatment of rats with CGRP can prevent the osteoporotic phenotype caused by ovariectomy[47], a finding which may be explained both by enhanced bone formation by CGRP as well as by decreased bone resorption.

Thus, the data obtained by adding the different peptides in the CT family of peptides to cells *in vitro*, or to rodents *in vivo*, have shown that CT inhibits bone resorption without having any effect on bone formation, whereas amylin and CGRP inhibit bone resorption and stimulate bone formation and adrenomedullin stimulates bone formation without having any effect on bone resorption.

Recently, further information on the role of the CT family of peptides *in vivo* has been obtained using mice with deletions of the genes for either CT/α-CGRP, amylin, *CTR* or α-*CGRP* and the phenotypes observed in these mice are unexpected when compared to previous observations obtained by administration of the peptides *in vivo* and *in vitro*.

Deletion of the genes encoding calcitonin/ α-calcitonin gene-related peptide and calcitonin receptor

It was anticipated that deletion of the *CT*/α-*CGRP* gene would lead to either no effect or to bone loss due to the lack of inhibition of bone resorption by CT. Surprisingly, *CT*/α-*CGRP*$^{-/-}$ mice have normal serum calcium and exhibit enhanced bone mass due to increased trabecular bone volume in both vertebral bodies and tibias[48]. The histomorphometric analysis showed that *CT*/α-*CGRP*$^{-/-}$ mice had increased trabecular number, no change in trabecular thickness, but reduced trabecular spacing. Similar observations were made in male and female mice. The number of osteoblasts was not affected by deletion of the *CT*/α-*CGRP* gene, but dynamic histomorphometry, using calcein double labelling, showed a 1.5- to 2-fold enhancement of bone formation rate. The number of osteoclasts was not significantly affected in the *CT*/α-*CGRP*$^{-/-}$ mice. However, osteoclast activity, as assessed by urinary excretion of deoxypyridinoline, was significantly increased in 1-month, but not 3-month-old mice, probably as a reflection of increased bone resorption caused by the lack of calcitonin.

Together, these data strongly suggest that the increased trabecular volume is due to increased bone formation rate and not due to decreased bone resorption. Interestingly, *CT*/α-*CGRP*$^{-/-}$ mice were resistant to ovariectomized - induced osteopenia because of increased bone formation rate. In the ovariectomized *CT*/α-*CGRP*$^{-/-}$ mice no enhanced number of osteoclasts was seen compared to sham operated *CT*/α-*CGRP*$^{-/-}$ mice, which could indicate that the preserved bone mass in these mice could also be due to inhibition of osteoclast formation. However, for reasons not explained, the wild type ovariectomized mice, although exhibiting decreased bone volume, did not show any increased number of osteoclasts which is usually observed in ovariectomized mice.

Although the increased bone formation rate was the most significant finding in *CT*/α-*CGRP*$^{-/-}$ mice, it was observed that under certain experimental conditions, the deletion of the *CT*/α-*CGRP* gene resulted in, as expected, increased bone resorption. Thus, *CT*/α-*CGRP*$^{-/-}$ mice showed significantly higher serum calcium after injection of either PTH or $1,25(OH)_2$-vitamin D_3, a response which could be reversed by CT, but not by α-CGRP. These observations indicate that the role of CT on bone resorption might be to function as an inhibitor under calcium stress. In fact, increased CT levels have been reported during pregnancy, growth and lactation[49].

These findings demonstrate that *CT*/α-*CGRP*$^{-/-}$ mice exhibit both an expected increased bone resorptive response and an unexpected enhanced rate of bone formation. It is not likely that the enhanced bone formation is caused by a lack of an inhibitory effect by CT or α-CGRP on osteoblasts, since the CTR is not expressed on osteoblasts and α-CGRP has been found to cause the opposite effect. Moreover, in a more recent paper, the same group show that mice with a selective deletion of the α-CGRP gene have decreased bone mass[36]. Currently, it is not understood how lack of CT signalling leads to enhanced bone formation. It seems, however, as if CTR signalling in wild type mice, either in osteoclasts or some other cell type expressing CTR, leads to subsequent events which, by a mechanism completely unknown, eventually causes decreased bone formation rate. Since CTR also are present in the brain[50], and since the central nervous system has been found to be able to regulate bone remodelling (see above), it

can not be ruled out that the skeletal phenotype in *CT*/α-*CGRP*$^{-/-}$ mice is mediated by the central nervous system. The information obtained so far shows that the neurons controlling bone remodelling are located in the hypothalamus, in which lack of leptin receptor signalling, or lack of NPY type 2 receptor signalling, cause enhanced bone mass. Mapping of CTR in the brain has demonstrated the expression of CTR in very many areas, but, interestingly, ventromedial, lateral and posterior hypothalamus, are three sites in which expression of immonoreactive CTR has been observed[51].

Confirming that lack of CT signalling via CTR leads to enhanced bone mass, it has recently been reported that heterozygous *CTR* knockout mice also exhibit increased bone mass due to enhanced bone formation rate[52].

It is interesting to note that similar to PTH, which was first identified as a major stimulator of bone resorption, but later found to be also a stimulator of bone formation, CT seems also to have dual effects on bone remodelling since CT now seems not only to be an inhibitor of bone resorption, but also an inhibitor of bone formation.

The role of CT as a physiological inhibitor of bone resorption has previously been questioned since thyroidectomized patients do not develop osteopenia and medullar thyroid carcinoma does not cause osteosclerosis. Based upon the high bone mass found in mice lacking the CT gene, it is now difficult to explain why thyroidectomized patients do not exhibit increased bone mass and patients with thyroid carcinoma do not exhibit decreased bone mass.

Selective deletion of the gene encoding α-CGRP

Since it is uncertain whether the increased bone mass in *CT*/α-*CGRP*$^{-/-}$ mice is caused by CT deficiency or α-CGRP deficiency, the same group which created the *CT*/α-*CGRP*$^{-/-}$ mice has now generated a mouse in which α-CGRP gene is selectively knocked out, with the CT gene still intact[36]. In contrast to *CT*/α-*CGRP*$^{-/-}$ mice, mice deficient in α-CGRP exhibit decreased bone mass. The mice had decreased trabecular bone volume, trabecular number and trabecular thickness and increased trabecular spacing. In contrast, cortical thickness was unaffected. The osteopenia was not due to enhanced bone resorption, since the number of osteoclasts and the excretion of deoxypyridinoline were unaffected. Static histomorphometry showed that also the number of osteoblasts, as well as the number of osteocytes, were unaffected. However, dynamic histomorphometry, using double calcein labelling, revealed that the rate of bone formation was substantially decreased. These findings are well in line with previous observations showing that CGRP is a stimulator of bone formation *in vitro* and *in vivo*[41-44]. The skeletal phenotype in *α-CGRP*$^{-/-}$ mice was not associated with any enhanced compensatory expression of CT, or changes in serum calcium or PTH. Confirming previous findings, it was also demonstrated that α-CGRP immunoreactive nerve fibers are present in the vicinity of trabecular bone. The authors also showed mRNA expression in primary mouse osteoblast cultures of the two components CRLR and RAMP1, which make up the CGRP receptor, in agreement with previous findings.

These observations show that it is the deficiency of CT in *CT*/α-*CGRP*$^{-/-}$ mice which causes the osteosclerotic phenotype and that the lack of α-CGRP results in osteopenia, indicating that the main effects of CT and α-CGRP are to regulate osteoblast activity, with CT being a physiological inhibitor and α-CGRP a stimulator of bone formation.

Although it remains to be proven that it is the role of α-CGRP as a signalling molecule in skeletal nerve fibers that causes the skeletal phenotype in α-CGRP deficient mice, it is most likely the explanation. This view fits into the observations that the sympathetic nervous system is able to regulate the rate of bone formation, since β-adrenergic signalling causes inhibition of formation and is suggested to mediate the antiosteogenic effect by leptin on the skeleton[5]. In line with this view, it was recently shown that capsaicin treatment destroys the unmyelinated sensory axons expressing CGRP and that the reduced CGRP signalling was associated with decreased bone mass[53], similar to patients with familial dysautomia which lacks unmyelinated sensory neurons and exhibit decreased bone mass and increased fracture rate. It should, however, be kept in mind that *α-CGRP*$^{-/-}$ mice still express β-CGRP and it will be interesting to know which will be the phenotype of *β-CGRP*$^{-/-}$ mice and of α-*CGRP*$^{-/-}$/β-*CGRP*$^{-/-}$ mice.

Deletion of the gene encoding amylin

Since the gene deletion approach had showed that the most important physiological role of CT is not to function as an inhibitor of bone resorption, it is reasonable to assume that other more important regulators exist. Recently, amylin, which both *in vitro* and *in vivo* has been found to inhibit bone resorption, was suggested to be a candidate as a physiological regulator of bone resorption. This view was based upon the observation that deletion of the amylin gene leads to low bone mass due to increased bone resorption[52]. Although osteoblasts express functional amylin receptors causing anabolic responses, *amylin*$^{-/-}$ mice did not show any sign of bone formation being affected. The low bone mass in amylin deficient mice was observed after 12 weeks of age, but not earlier, and expressed in the vertebrae and femurs of both male and female mice. The osteoporotic phenotype was caused by decreased trabecular and cortical thickness, unlike in *α-CGRP*$^{-/-}$ mice in which the low bone mass was restricted to decreased trabecular thickness. The loss of bone was not associated with any change in osteoblast number, or bone formation rate *in vivo*, nor in any differences in alkaline phosphatase activity, biosynthesis of type I collagen and mineralization of bone noduli *in vitro*. However, the urinary excretion of deoxypyridinoline was significantly enhanced already at 8 weeks of age and persisted for at least 24 weeks, which indicates that amylin deficiency leads to enhanced bone resorp-

Petides	*In vitro* experiments		*In vivo* experiments		Gene deletion experiments	
	Resorption	Formation	Resorption	Formation	Resorption	Formation
CT	↓	---	↓	---	(↓)	↓
Amylin	↓	↑	↓	↑	↓	---
CGRP	↓	↑	↓	↑	---	↑
Adrenom.	---	↑	↑	↑	?	?

Table 2. Summary of the function of peptides in the CT family of peptides on bone resorption and bone formation based on *in vitro* cultures, *in vivo* experiments using administration of the peptides and *in vivo* experiments using mice with gene deletions.

tion, which was supported by the observation that osteoclast number was enhanced 1.6-fold. The finding that amylin deficiency leads to an enhanced number of osteoclasts is likely due to an effect of amylin on osteoclast progenitor cell differentiation and/or fusion to multinucleated osteoclasts, since amylin was found to inhibit osteoclast formation in bone marrow macrophage cultures from wild type mice stimulated by RANKL and M-CSF, similar to previous findings showing that CT, CGRP and amylin can inhibit *in vitro* osteoclastogenesis in bone marrow cultures stimulated by 1,25$(OH)_2$-vitamin D_3[40]. The experiments do not prove, however, that amylin causes decreased osteoclast formation via receptors on osteoclast progenitor cells, since the bone marrow macrophage cultures are not pure cultures of osteoclast progenitor cells but may contain also other cells indirectly affecting osteoclastogenesis.

The most likely explanation is that amylin inhibits osteoclast formation due to activation of CTR associated with RAMPs. However, the observation that $CTR^{+/-}/amylin^{+/-}$ mice exhibit both increased bone formation and increased number of osteoclasts indicates that amylin is not dependent on CTR expression to cause decreased osteoclast formation, but may use a hitherto unrecognized receptor.

Summary

The CT family of peptides includes CT, CGRP, amylin, adrenomedullin and the two recently discovered peptides intermedin and CRSP. These peptides display a weak homology at the level of amino acid sequence, but are more closely related when the secondary structures are compared. The peptides are widely distributed in peripheral tissues, as well as in the peripheral and central nervous system and induce a large variety of biological effects, including on bone remodelling. CT, CGRP, amylin and adrenomedullin all exert effects on both bone formation and bone resorption, whereas effects by intermedin and CRSP on bone have not yet been studied. The CT family of peptides utilizes two different seven transmembrane G protein-coupled receptors - CTR and CRLR - of which the latter cannot bind ligands and may not be a proper receptor and was initially described as an orphan receptor. However, when it was found by an expressing cloning approach, that CRLR requires a single transmembrane protein called RAMP to function as a signalling receptor, the understanding of the receptors for the CT family of peptides made a great breakthrough. The RAMP family, consisting of three different RAMPs, can heterodimerize with both CRLR and CTR and it is now recognized that seven molecularly characterized receptors binding CT, CGRP, amylin and adrenomedullin exist. Activation of these receptors in cell cultures and in animal experiments have shown that CT, CGRP and amylin decrease bone resorption and that amylin, CGRP and adrenomedullin stimulate bone formation. However, when the skeletal phenotypes of mice with deletions of the genes encoding CT, CTR, α-CGRP and amylin have been examined it turned out that the physiological effects of these peptides are different from what could be anticipated from the gain of function studies (Table 2). Thus, the main role of CT seems to be as an inhibitor of bone formation with only a minor role on bone resorption. Amylin, on the other hand, seems to be a crucial inhibitor of bone resorption without any effect on bone formation, whereas CGRP has no effect on bone resorption, but is a stimulator of bone formation.

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Note added in proof: Recently, Davey et al. at the Twenty-seventh Annual Meeting of the American Society for Bone and Mineral Research reported that global knock down of the calcitonin receptor, in females six weeks of age, resulted in decreased bone mineral density, associated with decreased trabecular number but no change in mineral apposition rate or osteoclast surface, suggesting that the phenotype is due to increased bone resorption because of enhanced osteoclast activity rather than enhanced osteoclast formation. No changes in any of the histomorphometric parameters were observed in young male mice [J Bone Miner Res 20 (Suppl. 1): abstr 1161, 2005.

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