

Review

Calcitonin: The Other Thyroid Hormone

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Calcitonin was originally discovered as a hypocalcemic factor synthesized by thyroid parafollicular C cells. Early experiments demonstrated that calcitonin inhibited bone resorption and decreased calcium efflux from isolated cat tibiae and subsequent histologic and culture studies confirmed the osteoclast as its major site of action. Its potent antiresorptive effect and analgesic action have led to its clinical use in treatment of Paget's bone disease, osteoporosis, and hypercalcemia of malignancy. This review surveys the cellular and molecular basis of these physiologic and clinical actions.

Introduction

THYROID PARAFOLLICULAR CELLS (C cells) are the major producers of circulating calcitonin (1–4), a potent anti-resorptive hormone (5–8). In this short review, we will discuss its synthesis, molecular action, and use as a drug for diseases characterized by increased osteoclastic bone resorption (9,10).

Synthesis and Structure

The calcitonin gene complex comprises two known genes, α and β genes (for review see [6,11]). The α calcitonin gene has six exons; the first three are shared with the alternative splice product (12) a potent vasodilator and neuropeptide, calcitonin gene-related peptide (CGRP). When spliced at exon 4 this yields calcitonin; splicing at exon 6 produces CGRP (13). The β gene is similarly organized but has differing 3' and 5' noncoding regions (14) and only gives rise to β -CGRP. The calcitonin promoter that regulates calcitonin expression possesses a negative response element to vitamin D, a cyclic adenosine monophosphate (cAMP)-response element (CRE) and a transcriptionally active octamer sequence (CRE-O) (15,16). After splicing to either calcitonin or CGRP mRNA, mature peptides are synthesized initially as large precursors that are cleaved intracellularly to release the active molecule (Fig. 1).

The mature peptide contains 32 amino acids. It possesses an N-terminal disulfide bridge between residues 1 and 7; the latter is positioned between residues 2 and 7 in CGRP (17). There is a proline amide at residue 32. Calcitonin sequences vary between species but 8 residues (at positions 1, 4, 5, 6,

7, 9, 28, and 32) are conserved in all species studied so far. Of these, 6 cluster at the N-terminus; the remaining 2 occur at the ends of the molecule. Circular dichroism (CD) spectroscopy demonstrates that human calcitonin forms a left-handed extended helix in solution (18). Recent studies of synthesized lactam bridge-containing biologically active calcitonin derivatives implicate the amphiphilic α -helix and a type 1 β -turn (involving residues 17–20) in receptor recognition by calcitonin (19). It has been suggested that the conformational flexibility of a given calcitonin molecule depends on bulkiness of the relevant side chains and determines its biologic potency (for review see Breimer et al. [20]). For example, three relatively compact conserved glycine residues occur in the highly potent salmon and eel calcitonins. Furthermore, a number of modifications in primary structure that include deletion of the C-terminal proline amide, shortening of the C-terminal end, cleavage of the disulphide bond, and oxidation of the human calcitonin methionine-8 reduce biologic activity. Conversely, increases in homology with salmon calcitonin generally increase biologic activity.

Cellular and Molecular Actions of Calcitonin

Calcitonin inhibits basal and stimulated resorption of organ cultured intact bone (for review see Breimer et al. [20]). It rapidly causes loss of the ruffled border of osteoclasts in bone sections (21–23). Near-physiologic, femtomolar calcitonin concentrations halt cytoplasmic motility and produce gradual pseudopodial retraction in isolated osteoclasts (7). Such effects have been resolved into an early cessation of

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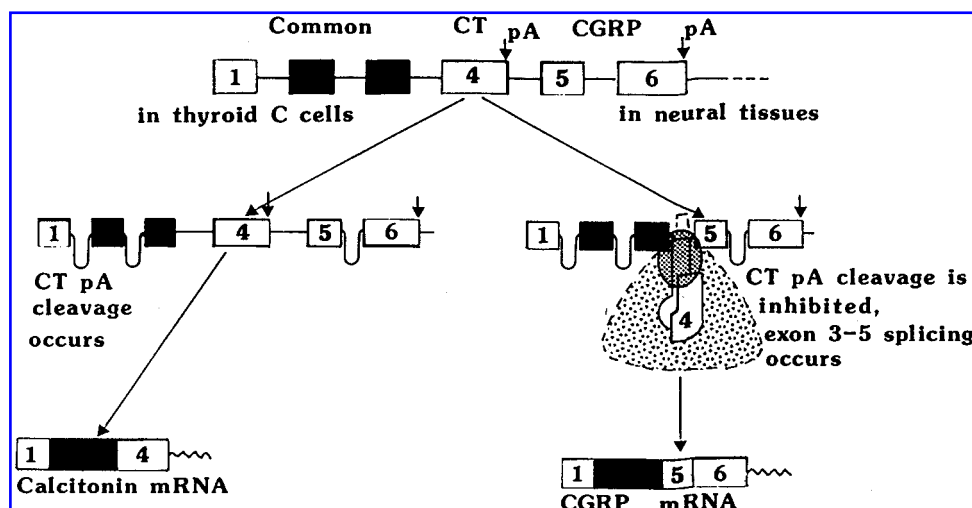


FIG. 1. Model for calcitonin gene expression.

motility or quiescence (Q) component with a half-time, $t_{1/2}$, of 15 minutes, and a later pseudopodial and margin retraction (R) with a $t_{1/2}$ of 27 minutes. Both changes decrease the area of cell contact with bony substrate (17,24,25). Calcitonin also inhibits synthesis and release of tartrate-resistant acid phosphatase (TRAP) by osteoclasts (26). It also alters both Na^+ - K^+ -ATPase activity and carbonic anhydrase localization and reduces acid secretion (27–29). Such cellular effects of calcitonin correlate well with results from osteoclast bone resorption assays (8,30,31) as quantified through pit number or the area or volume of bone resorbed. The different calcitonins display a rank order of *in vitro* inhibitory potency that correlate closely with *in vivo* potency estimates with salmon calcitonin being the most and human the least potent (32; Fig. 2). CGRP and amylin also interact with the calcitonin receptor but only at 100- and 40-fold higher concentrations (8,24,32,33).

Calcitonin also affects other tissues indirectly related to bone remodeling. It increases mRNA expression of the CYP27B1 enzyme 25-hydroxyvitamin D_3 1α -hydroxylase (CYP27B1); this catalyses biosynthesis of 1α , 25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3 in renal proximal tubules (34). It also alters the localization of critical ion exchangers in renal tubular cells (35).

Calcitonin also interacts with osteoblasts in some systems in the apparent absence of calcitonin receptors. Thus, eel calcitonin exerts an anabolic effect on osteoblasts that enhances osteoinduction by BMP-2 (36,37). It also increases the concentration of insulin-like growth factors in serum-free cultures of human osteoblast-like cells (38). Calcitonin may also prevent osteoblast and osteocyte apoptosis but this action is controversial (39), and unlikely real.

Additional cellular actions of calcitonin include its effect on the growth of breast cancer cells. Calcitonin retards cell division in calcitonin receptor transfected HEK cells. New evidence suggests that calcitonin can affect the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 that arrests cell cycle in the G_1 and G_2/M phases (40). More interesting is the observation that calcitonin suppresses expression of the growth factor, PTHrP, in breast cancer cells. Such developing evidence for relations between the effects of calcitonin

and of those classes of cell cycling molecules, growth factors, and cell growth revives a long-forgotten area of calcitonin and cancer biology. Furthermore, calcitonin inhibits the osteoclastogenic effects of rank-ligand, an effect that may be relevant therapeutically (41). Finally, pharmacologic calcitonin concentrations increase renal calcium and phosphate excretion and 1,25-dihydroxycholecalciferol production (42). More recently, it has been shown to be a major regulator of the expression of the renal 25-hydroxyvitamin D_3 hydroxylase gene in normocalcemic rats (43).

Calcitonin Receptors and Signal Transduction

Several calcitonin receptor subtypes have been cloned and sequenced (44). All calcitonin receptors bind calcitonin with high affinity and the alternative ligands, CGRP, amylin, and adrenomedullin with lower affinity. However, several groups have cloned calcitonin receptor-like receptors that primarily bind CGRP and amylin (for review see Wimalawansa [45]). All calcitonin receptors belong to the PTH/PTHrP/calcitonin/adrenomedullin/secretin receptor superfamily with seven transmembrane domains and a long extracellular domain (for review see Zaidi et al. [25]). The deduced peptide sequences exhibit multiple hydrophobic domains flanked by charged residues. There are six Cys residues in the extracellular loops and two N-linked glycosylation sites. Both structures are well conserved and may be major requisites for ligand binding (46; Fig. 3).

Human calcitonin receptor subtypes arise from alternative splicing of the primary mRNA transcript of the human calcitonin receptor gene (47–49) that consists of multiple exons separated by lengthy introns allowing for splicing (50). The two most common human subtypes isolated from BIN-67 and T47D cells respectively differ by a 16 amino acid insert in the first putative intracellular domain (51,52). The most prevalent insert-positive C1a form can transduce intracellular signals at least through G_s and G_q protein-coupled mechanisms (53,54). The two C1a and C1b rat isoforms cloned from osteoclasts differ in the structure of their first extracellular loop (55,56).

There are approximately 1 million calcitonin receptors per

	Man1	Rat	Sa.2	Sa.3	Sa.1	Eel	Chick	Porc.	Bov.	Ov.	Man2*
H 											
1 Cys											Tyr
2 Gly		Ser	Ser	Ser	Ser	Ser	Ala	Ser	Ser	Ser	Ser
3 Asn							Ser				
4 Leu											
5 Ser											
6 Thr											
7 Cys											
8 Met			Val	Val	Val	Val	Val	Val	Val	Val	Leu
9 Leu											Gln
10 Gly								Ser	Ser	Ser	
11 Thr		Lys	Lys	Lys	Lys	Lys	Ala	Ala	Ala		
12 Tyr		Leu	Leu	Leu	Leu	Leu					
13 Thr		Ser	Ser	Ser	Ser	Ser	Trp	Trp	Trp	Trp	Leu
14 Gln							Arg	Lys	Lys		
15 Asp				Glu	Glu	Glu	Asn				Tyr
16 Phe	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
17 Asn		His	His	His	His	His					Lys
18 Lys							Asn	Asn	Asn	Asn	Asn
19 Phe		Leu	Leu	Leu	Leu	Leu		Tyr	Tyr		
20 His		Gln	Gln	Gln	Gln	Gln					
21 Thr				Tyr	Tyr	Tyr	Arg	Arg	Arg	Arg	Met
22 Phe						Ser					
23 Pro				Arg	Arg	Arg	Ser	Ser	Ser	Ser	
24 Gln		Arg	Arg	Arg	Arg	Arg	Gly	Gly	Gly	Gly	Gly
25 Thr							Met	Met	Met	Met	Ile
26 Ala	Ser	Asn	Asn	Asn	Asp	Asp	Gly	Gly	Gly	Gly	Asn
27 Ile		Thr	Thr	Thr	Val	Val	Phe	Phe	Phe	Phe	Phe
28 Gly											
29 Val		Ala	Ala	Ser	Ala	Ala	Pro	Pro	Pro	Pro	Pro
30 Gly						Glu	Glu	Glu	Glu	Glu	Gln
31 Ala		Val	Val	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Ile
32 Pro											
NH2											

* Man2 - predicted

FIG. 2. Amino acid sequence of the calcitonins.

rat osteoclast (57). However, little is known about the regulation of calcitonin receptor expression. There is recent evidence that calcitonin itself inhibits calcitonin receptor expression at the transcriptional level (58), that glucocorticoids stimulate calcitonin receptor expression (59) and that osteoclast calcitonin receptor expression is downregulated during osteoclastogenesis (60). It has also been suggested recently that macrophage colony-stimulating factor (M-CSF) and nuclear factor- κ /B (NF κ B) regulate calcitonin receptor expression and function in human osteoclast-like cells (61). Calcitonin receptors can also be induced in mammary tissue during pregnancy (62) but the physiologic significance of this observation is unclear. Finally, the transgenic expression of calcitonin receptor in mice indicates its role in morphogenesis in general, and skeletal development in particular (63).

There were early suggestions that identified osteoclast calcitonin receptors with Ca^{2+} sensing receptors but it was subsequently demonstrated that such evidence reflected the cation-sensitive nature of calcitonin binding (64). Thus, it is now becoming clear that osteoclast Ca^{2+} sensing is mediated instead by a cell surface ryanodine receptor controlled by

cyclic adenosine diphosphate (ADP)-ribose generated from the ADP-ribosyl cyclase CD88 (65-67). Nevertheless, nuclear membrane ryanodine receptors and CD88 in bone cells provide a potential mechanism through which cytosolic Ca^{2+} changes such as those triggered through calcitonin receptor activation are transduced intranuclearly to affect gene transcription (68,69). There is thus the possibility that cellular mechanisms related to the calcitonin and Ca^{2+} receptor systems in the osteoclast interact at several levels of cellular organization.

Studies using chimeric receptor constructs and site-directed mutagenesis have further advanced our understanding of calcitonin receptor biology. Use of chimeric receptors between the calcitonin receptor and the insulin-like growth factor receptor made it possible to identify the molecular regions required for G_s -mediated signal transduction particularly in the third intracellular loop (70). Deletion of 14 amino acids in the seventh transmembrane domain both abolished coupling to phospholipase C and altered ligand binding (71). Modifications in the first intracellular domain and N-terminus also influenced both ligand binding and signal trans-

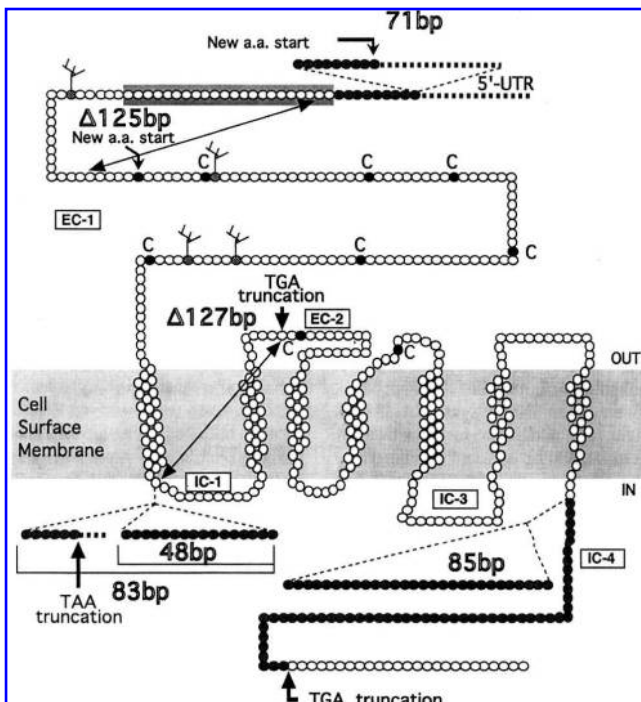


FIG. 3. Model for calcitonin receptor expression. (Galson DL, Goldring SR Structure and Molecular Biology of the Calcitonin Receptor. In: Bilezikian, Raisz, Rodan (eds). *Biology of Bone*, 2nd Ed., Academic Press, 2002, vol. 1 p605.) Reproduced with permission from Academic Press.

duction (72). Finally the use of chimeric receptors between the calcitonin and glucagon receptors demonstrated two dissociable binding sites (73). Such strategies together thus hold promise for clarifying important tertiary structural prerequisites for the interaction between calcitonin and its receptor.

Pharmacologic studies on intact osteoclasts separate distinct signal transduction pathways that could involve distinct calcitonin receptor subtypes (17). Different calcitonin receptor isoforms couple to the G_s , G_q , or G_i protein systems (51,53,74,75). This can also take place in a cell cycle-dependent manner with divergent structural requirements (35,55). A cholera toxin-sensitive G_s protein mediates the cAMP-dependent Q (quiescence) component of the osteoclast motility response (see above) whereas a pertussis toxin-sensitive G_i protein mediates the R (retraction) component that depends on increases in cytosolic Ca^{2+} . Whereas the Q component is also activated by related peptides, including amylin and CGRP, the R effect is highly specific for calcitonin (75–78). However, receptor activity modifying proteins (RAMPs) can form heterodimers with two G protein-coupled receptors to alter the ligand-specificity of a given receptor (79,80). RAMP-1 interacts with human calcitonin receptor Clb as well as the calcitonin receptor-like receptor. RAMP-3 interacts with the calcitonin receptor and enhances its binding to amylin (81).

Inhibiting or depleting protein kinase C spares the activation of adenylyl cyclase with the G_s -coupled response that is thought to trigger the Q effect. This is consistent with its involvement instead with a protein kinase A pathway (82) that involves the third intracellular loop and C-terminal tail

of the calcitonin receptor (70). In contrast, protein kinase C activation may upregulate G_i -coupled signaling (83). Signaling through G_q activates both phospholipase C and protein kinase C in osteoclasts. This activation with resulting phosphorylation of *Shc* and ERK1/2 as well as the human enhancer of filamentation (Hef-1 or CasL) likely induces pseudopodial retraction or the R effect (84). Finally, calcitonin receptor activation also triggers phospholipase D signaling although with at present unknown physiologic consequences (85).

Calcitonin may also regulate osteoclast adhesion mechanisms important in pseudopodial retraction. Pyk2 is a major adhesion-dependent tyrosine kinase in osteoclasts that associates with c-Src via its SH2 domain. Engagement of the major, $\alpha_v\beta_3$, integrin present in osteoclasts, increases tyrosine phosphorylation of Pyk2, and leads to the formation of the sealing zone (86). Hef-1 or CasL is a focal adhesion-associated, multiple-domain docking protein that contains an SHB domain that binds focal adhesion kinase (FAK) and Pyk2. Transduction from the calcitonin receptor that activates Hef-1 but not p130^{cas} depends on protein kinase C, and thus the G_i -coupled calcitonin receptor, but is independent of the G_s /cAMP/protein kinase A pathway (87). Nevertheless, integrin engagement, actin cytoskeletal rearrangement and c-src activation are required for calcitonin-induced tyrosine phosphorylation of paxillin and HEF1, but not for Erk1/2 phosphorylation (88). Activation of HEF-1 by calcitonin may therefore cause interactions with Pyk2 and other molecules that regulate osteoclast adhesion, although this remains to be established.

Physiologic Role of Calcitonin

The exact physiologic role of calcitonin has not been established in humans. Its role in calcium regulation may be confined to times of stress, such as growth, pregnancy, lactation (89).

Current and Future Therapies Using Calcitonin

Several prospective controlled trials have reported that calcitonin stabilizes or modestly increases mineral density in trabecular, but not cortical bone. Such findings were made in osteoporotic patients treated for 5 years or less (90–94) and in situations where it was used to prevent menopausal trabecular bone loss (92,95). Calcitonin also significantly reduces the incidence of hip fracture, vertebral compression fracture, and peripheral limb fractures in both postmenopausal and elderly women (93,96). Preliminary data from the PROOF trial, a 5-year double-blind, randomized, placebo-controlled study of 1255 postmenopausal women with osteoporosis showed that 200 IU of salmon calcitonin nasal spray reduces the risk of vertebral fractures by 36% but failed to reduce hip fracture. The benefit in fracture risk at even 12–18 months preceded long-term changes in bone mineral density, and might be attributed to changes in trabecular microarchitecture influencing the pathogenesis of vertebral fractures (97). Recent clinical trials with calcium plus vitamin D, calcitonin, and raloxifene indicate significant protection from fracture despite only modest increases in bone mineral density possibly through an effect on conserving bone microarchitecture (98).

More widespread clinical usage of calcitonin (99) is lim-

ited almost purely by bioavailability (100). However, there is the possibility of several new approaches to enhance calcitonin access to the osteoclast. First, novel allosteric activators of the calcitonin receptor might make the receptor more sensitive to circulating calcitonin (101). Second, there is the possibility for the development of an oral calcitonin coupled to a molecule that might ensure improved bioavailability (100–104). These compounds are also likely to be absorbed in a site-specific manner, for example, in the colon (102) or possibly through pulmonary and transdermal routes (105,106). There is the possibility of a polyethylene glycol-modified salmon calcitonin that has significant biologic activity and a prolonged plasma half-life (107). Finally, there are now gene therapy vectors available that would allow osteoclast precursors to carry the calcitonin gene to specific bone sites of osteolysis. The next few years should therefore see calcitonin revived as an important therapeutic agent (108).

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