REVIEW ARTICLE

Peptides from the calcitonin genes: molecular genetics, structure and function

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INTRODUCTION

It is increasingly recognized that normal cellular function is dependent on a number of complex interacting regulatory systems, not least in multicellular organisms which develop from a single fertilized egg. The study of the calcium regulation hormone, calcitonin, illustrates these principles. The calcitonin/calcitonin gene-related peptide (CGRP) gene complex encodes a small family of peptides: calcitonin, its C-terminal flanking peptide, katacalcin and CGRP. Whereas calcitonin maintains the skeleton during periods of calcium stress, the function of katacalcin, if any, is still unknown. The possible existence of CGRP was predicted for the rat by the application of recombinant DNA technology (Rosenfeld *et al.*, 1983), and its presence subsequently established in man (Morris *et al.*, 1984). The genetics of calcitonin/CGRP has been reviewed (Rosenfeld *et al.*, 1984; Leff *et al.*, 1986; Zaidi *et al.*, 1987a). Here we concentrate on certain novel aspects of gene organization, expression and splicing, and focus on recent developments in the understanding

of the structure and function of the peptides encoded by the genes. The steps involved in the formation of a primary transcript, including promoter selection, termination 3'-end formation, capping and polyadenylation are beyond the scope of this review (see Rosenfeld *et al.*, 1984; Platt, 1986; Leff *et al.*, 1986; Padgett 1986).

MOLECULAR GENETICS

Gene organization

The calcitonin/CGRP gene complex comprises at least two genes, which are commonly referred to as the α and β genes. Having been discovered first, the α gene is better understood than the β gene. In man, both genes are located on the short arm of chromosome 11, between the catalase and parathyroid hormone genes (Kittur *et al.*, 1985; Hoppener *et al.*, 1985; Alevizaki *et al.*, 1986) (Fig. 1). The α -calcitonin/CGRP gene stretches over approx. 6.5 kb and is transcribed in full. The gene consists of six

Fig. 1. The structural organization of the α -calcitonin/calcitonin gene-related peptide gene, in a schematic representation

From the same primary transcript, which has two polyadenylation sites, two different mRNAs may be produced, one coding for the calcitonin precursor and the other coding for the CGRP precursor. The differential tissue-specific processing involves the selective use of the polyadenylation site combined with cleavage and splicing. The organization of the β -gene is similar; the 5' and 3' non-coding regions exhibit 40% divergence, the CGRP coding region has > 90% similarity and the exon IV-like region is 65% similar. (Reproduced with kind permission from Q. J. Exp. Physiol.)

Abbreviations used: CGRP, calcitonin/calcitonin gene-related peptide; MTC, medullary thyroid carcinoma; PTH, parathyroid hormone.

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exons: the first three exons are present in both calcitonin and CGRP mRNA, though exon I is not translated. Exon IV contains the calcitonin-coding sequence and a sequence encoding its C-terminal flanking peptide, katalcalcin. This is followed by an untranslated sequence, at the end of which there is the usual polyadenylation signal AATAAA (Proudfoot & Brownlee, 1976), used for generating calcitonin mRNA. Exon V encodes the CGRP sequence. The sixth exon, which is also part of the CGRP transcript, is not translated. It ends with an alternative polyadenylation signal ATTAAG (Jung *et al.*, 1980). It has been shown that cells synthesizing calcitonin splice together exons I–IV to form mature mRNA, whereas cells which produce CGRP splice together exons I–III, V and VI (Rosenfeld *et al.*, 1981, 1982; Amara *et al.*, 1982; Craig *et al.*, 1982; Jonas *et al.*, 1985). Since the first three exons are spliced in either process, both calcitonin and CGRP mRNA have identical 5' coding and non-coding regions.

The peptides are synthesized as large precursors that are cleaved intracellularly to release the active molecule (Goodman *et al.*, 1979; Jacobs *et al.*, 1979, 1981a; Amara *et al.*, 1980; Allison *et al.*, 1981). The first 25 amino acids constitute the leader sequence (or signal peptide) that determines secretion (Walter *et al.*, 1984; Wickener & Lodish, 1985). This sequence is removed by a microsomal membrane-associated enzyme (Jacobs *et al.*, 1981b). The sequences of calcitonin and CGRP diverge exactly at a genomic splice junction but continue with open reading frames. Nevertheless, the same organization is essentially retained in that each active peptide is flanked by highly conserved basic residues: a pair of Lys-Arg residues at the N-termini, and at the C-termini a Gly-Lys-Lys-Arg sequence in the case of calcitonin, while a Gly-Arg-Arg-Arg sequence flanks the CGRP sequence. These sequences serve as signals for post-translational processing (Douglas *et al.*, 1984). The glycine residue serves as an amino donor for amidation, in common with other peptides that are amidated at the C-terminus, such as corticotropin (Birnbaum *et al.*, 1982; Land *et al.*, 1982; Bradbury *et al.*, 1982). It has been reported that such processing is aberrant in certain tumours (Zajac *et al.*, 1985; Riley *et al.*, 1986). Also, the predicted sequences for calcitonin and CGRP are better conserved than those of non-functional peptides encoded by other regions of the gene.

The organization of the β gene is similar to that of the α gene, and it is likely that the two genes may have arisen by duplication. However, both 3' and 5' non-coding regions of the two genes diverge significantly. Whereas exons III (common-coding) and V (CGRP-coding) are similar (about 95% similar), exon VI is only somewhat alike (65% similar). This divergence has permitted the generation of cDNA probes specific for the two genes (see below). In the rat and in man, the α and β sequences differ by one and three amino acids respectively; two of these changes occur at positions similar to those altered in rat α -CGRP as compared to the corresponding human sequence (see Fig. 3).

There is no evidence for the production of calcitonin mRNA from the β gene. However, there have been conflicting reports concerning the existence of calcitonin-related areas within the gene (Jonas *et al.*, 1985; Steenbergh *et al.*, 1985). Cloning and sequence analysis of the exon IV-like region of the human β gene has revealed that it is unlikely that a second calcitonin-like

peptide is expressed (Alevizaki *et al.*, 1986; Steenbergh *et al.*, 1986). The deduced reading frame terminates after only eight amino acid residues, and the sequences involved in the formation of the polyadenylated 3'-end are substantially altered, including the AATAAA sequence which has been replaced by AATCAT. Indeed, there is only 67% sequence identity between the exon IV-like regions of the two genes, which is comparable to that observed between the CGRP non-coding regions of the two genes, and is in sharp contrast to the highly conserved CGRP-coding regions. Nevertheless, a 33-amino-acid-long sequence, which is the part of a potential alternative open reading frame, shares considerable homology with salmon, human and bovine calcitonin. It can, however, not form the usual ring structure because the N-terminal cysteine is replaced by a tyrosine residue. Moreover, neither proteolytic processing signals nor an alternative splice acceptor site can be deduced that may allow the expression of a second calcitonin. It appears, therefore, that there has been no selective pressure to maintain the integrity of this segment of the β gene. Perhaps the β gene allows the expression of CGRP at sites or in circumstances where the inadvertent production of calcitonin could be deleterious.

Pattern of gene expression

Using calcitonin- and CGRP-specific cDNA probes, it has been shown that the alternative production of calcitonin or CGRP mRNA is regulated tissue-specifically (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). The pattern of mature transcripts corresponds to the immunochemical analysis of calcitonin and CGRP expression (for review, see Zaidi *et al.*, 1987a). Calcitonin mRNA is found almost exclusively in the thyroid and CGRP mRNA in the nervous system (Rosenfeld *et al.*, 1983; Sabate *et al.*, 1985). However during aberrant expression of the genes in medullary thyroid carcinoma (MTC), higher levels of CGRP mRNA expression have been noted. CGRP mRNA has been localized to a large number of sites in the central and peripheral nervous system (for review, see Zaidi *et al.*, 1987a). The highest levels of mRNA expression have been identified in the trigeminal ganglia, while lower amounts have also been noted in the hypothalamus, lateral medulla and motoneurons of the spinal cord. CGRP mRNA levels of the trigeminal ganglia approach those of prolactin- or growth hormone-specific mRNA in the pituitary (Sabate *et al.*, 1985), which suggests a primary physiological role of CGRP in cerebral blood flow homeostasis (McCulloch *et al.*, 1986; see below).

The differential expression of the α - and β -CGRP genes in the nervous tissue has been studied by *in situ* hybridization using cDNA probes specific for the respective genes (Amara *et al.*, 1985). There is evidence of some differential distribution, but its significance is unclear. For example, there is more β - than α -CGRP mRNA in the motor nuclei of the III, IV and V cranial nerves. The reverse is the case with the trigeminal ganglia, which contain at least 10-fold more α - than β -CGRP mRNA. Hybridization signals for both α - and β -CGRP mRNA are equivalent in VII, X and XII cranial nerves (Amara *et al.*, 1985).

Regulation of gene expression

The genes responsible for maintaining correct endocrine cell differentiation and regulation of calcitonin

production in the thyroid C-cells are not known. Their expression may be altered either as an initial event or as a consequence. Generally, in medullary thyroid carcinomas, the parent C-cell type is maintained and calcitonin is the main product (Amara *et al.*, 1982; Rosenfeld *et al.*, 1984; Nelkin *et al.*, 1984; Jonas *et al.*, 1985). However, occasionally there is switching to a low calcitonin state which coincides with high CGRP production: this led to the discovery of CGRP (Rosenfeld *et al.*, 1981). Moreover, low calcitonin production occurs with an apparent loss of C-cell differentiation in patients dying of metastatic MTC (Trump *et al.*, 1979; Saad *et al.*, 1984). Thus dedifferentiation appears to be associated with the switching to a low-calcitonin/high-CGRP state.

Differentiation properties have been induced in a cultured MTC cell line (TT) by agents known to activate protein kinase C, such as phorbol esters, or protein kinase A, such as cyclic AMP (deBustros *et al.*, 1985, 1986). The effects of these two agents in combination appear to be additive rather than synergistic. The morphological changes were found to be accompanied by inhibition of cell proliferation, increased secretion of calcitonin and enhanced calcitonin and CGRP mRNA production. Thus, the treatment did not effect the splicing pattern. In addition, *c-myc* transcription and DNA synthesis was substantially depressed. These changes in gene expression and hormone secretion were found to mimic those reported for a number of other peptides (Vale & Rivier, 1977; Virji *et al.*, 1978; Osbourne & Tashjian, 1981; Murdoch *et al.*, 1982; Malaisse *et al.*, 1983; Peterfreund & Vale, 1983; Supowit *et al.*, 1983). [The mediation of the response to phorbol esters is a complex process, but is thought to involve phosphorylation of unidentified regulatory protein molecules (Chiu *et al.*, 1987).]

The same group now report that the introduction of the viral Harvey *ras* (v-Ha-*ras*) oncogene into these cells induces similar differentiation properties enhances calcitonin/CGRP gene expression and inhibits cell proliferation and DNA synthesis (Nakagawa *et al.*, 1987). Expression of v-Harvey-*ras* is associated with a marked effect on the splicing pattern. There is a clear shift in favour of calcitonin, rather than CGRP mRNA production. This feature is in marked contrast to that seen with phorbol esters and cyclic AMP, and presumably represents the activation of an alternative transduction pathway. It is generally held that the v-Ha-*ras* gene product activates protein kinase C by increasing cellular levels of diacylglycerol (Barbacid, 1987). It has been documented that cells developmentally similar to human MTC cells, such as rat pheochromocytoma (PC12) cells, exhibit a similar differentiation pattern when transfected with the v-Ha-*ras* gene or microinjected with activated c-Ha-*ras* protein. These effects appear to be independent of cyclic AMP activation (Lacal *et al.*, 1984; Noda *et al.*, 1985). It is, however, not clear whether v-Ha-*ras* induces any change in the levels of *c-myc* expression or alters the state of methylation of the endogenous calcitonin genes. Indeed, the authors have previously reported that the 5' end of the α -calcitonin/CGRP gene is hypomethylated in MTC cells and is hypermethylated in human lung cancer and lymphoma (Baylin *et al.*, 1986). Indeed this work suggests a role of the *ras* protein in neuroendocrine differentiation.

Proposals that the expression of the calcitonin/CGRP

gene is regulated at the level of transcription by vitamin D and post-transcriptionally by calcium (Segond *et al.*, 1985) have yet to be confirmed. The doses of vitamin D used were not physiological. There has also been an isolated report that there could be a corticosteroid receptor sequence near the gene (Muszynski *et al.*, 1983).

Alternative splicing and its regulation

Alternative splicing is the joining of different combinations of exons from a primary transcript to give mature mRNAs that encode different proteins. It is one of several strategies that eukaryotic cells use to diversify their gene products. More than 50 genes, ranging from *Drosophila* to man, are known to generate protein diversity through alternative splicing (Breitbart *et al.*, 1987). Alternative splicing of pre-mRNA offers a rapid and reversible response in terminally differentiated cells that have lost the power to synthesize and rearrange DNA. It also indicates that consensus splice sites are not equivalent, and that there must be other regulatory factors. The factors which regulate the pattern of alternative splicing in various genes are not understood: sequences in the primary transcript (*cis*-acting factors) are necessary, but are not sufficient, and external (*trans*-acting) factors must also be involved. In the case of the α -calcitonin/CGRP gene, it was originally proposed that the pattern was regulated at the level of polyadenylation selection (Amara *et al.*, 1984). However, it is now clear that the splicing together of the exons generally precedes polyadenylation (Bovenberg *et al.*, 1986).

The pattern of expression of a series of constructs of the rat α -calcitonin/CGRP gene in heterologous cell lines and transgenic mice has recently been reported (Leff *et al.*, 1986; Crenshaw *et al.*, 1987). It has been shown that alteration of the calcitonin poly(A) site at the end of exon IV does not affect cells producing CGRP mRNA. However, cells mainly producing calcitonin stop expressing mRNA for both peptides. Instead, there is accumulation of partially processed nuclear transcripts: these are polyadenylated at the CGRP poly(A) site and contain the third and fourth introns. It appears that poly(A) site-selection is not regulated because cells transfected with constructs lacking the upstream splice donor sites of either exon IV or V acceptor have mature transcripts ending at either poly(A) site [most of these are, somewhat surprisingly, polyadenylated at the first (calcitonin) site]. In addition, when tandem constructs of the poly(A) sites for calcitonin or CGRP are substituted for the normal poly(A) site in a vector, the foreign sites are used at random. On the other hand, a deletion in the third intron (with the 3' deletion border coinciding exactly with the exon IV splice acceptor site) has been found to abolish the production of mature calcitonin mRNA. CGRP mRNA is still produced, although inefficiently. Furthermore, the deletion of 550 bp covering a large portion of the fourth exon and intron, including the calcitonin poly(A) site, abolishes not only calcitonin but also CGRP mRNA production. Thus, sequences important for splicing appear to reside in this fragment.

Recently, Rosenfeld's group also raised transgenic mice expressing the rat α -calcitonin/CGRP gene under the control of the mouse metallothionein promoter (Crenshaw *et al.*, 1987). They concluded that most cells that do not express the endogenous calcitonin/CGRP gene, nevertheless, make a clear splicing choice. In the

majority of tissues studied 90–97% of the transgene encodes calcitonin; the level of expression is highest by far in the liver and kidney. However, both calcitonin and CGRP mRNAs have been detected in the brain. It has also been shown, using immunohistochemistry and *in situ* RNA hybridization, that, while CGRP transcripts are selectively expressed in most neurones, calcitonin mRNA is found only in certain non-neuronal structures. Thus it appears that the choice of splicing operates independently of gene transcription.

On the basis of these studies a new model for the regulation of alternative splicing has been proposed (Leff *et al.*, 1986). Briefly, splicing to calcitonin mRNA is constitutive. In certain neurones a 'splice commitment' regulatory factor (or factors) is synthesized. This modulates pre-mRNA secondary structure and leads to the splicing of exon III to exon V, as the critical event in CGRP mRNA production. A similar model has been independently proposed by the Hammersmith group (Zaidi *et al.*, 1987a). The nature of this hypothetical factor (or factors) is not known. Although studies *in vitro* have suggested that alternative splicing could be induced by pre-mRNA secondary structure (Solnick, 1985), it is now clear that most pre-mRNA secondary structures are either not formed at all or else, are not maintained long enough to influence the choice of splicing *in vivo* (Solnick & Lee, 1987). A number of models of splicing have been proposed, which have, in common, the binding of small nuclear ribonucleoprotein complexes (snRNPs) to the pre-mRNA (Konarska & Sharp, 1986; Sharp, 1987; Chabot & Steiz, 1987; Bindereif & Green, 1987; Maniatis & Reep, 1987). Possibly, the specificity of the splicing pattern could reside in small RNA molecules, such as U1 (Zhuang *et al.*, 1987). For example in *Xenopus laevis*, the expression of various U1 snRNA species is developmentally regulated (Forbes *et al.*, 1984).

STRUCTURE

Comparative chemistry

Calcitonin is a 32-amino-acid single-chain polypeptide, with an *N*-terminal disulphide bridge (between positions 1 and 7) and a *C*-terminal proline amide residue. Several mammalian and submammalian calcitonins have been isolated, sequenced and synthesized: porcine (from normal thyroid: Baghdiantz *et al.*, 1964; Potts *et al.*, 1968; Rittel *et al.*, 1968); human (from medullary thyroid carcinoma tissue; Riniker *et al.*, 1968; Neher *et al.*, 1968); bovine (from normal thyroid: Brewer & Ronan, 1969); ovine (from normal thyroid: Potts *et al.*, 1970); salmon (from ultimobranchial bodies: O'Dor *et al.*, 1969; Niall *et al.*, 1969); eel (from ultimobranchial bodies; Otani *et al.*, 1976; Noda & Narita, 1976; Morikawa *et al.*, 1976); rat (from normal rat thyroid; Raulias *et al.*, 1976; from medullary thyroid carcinoma tissue: Byfield *et al.*, 1976a,b). The sequences of calcitonins from eight species are shown in Fig. 2. Generally, these are classified under three chemical types: rat/primate type (human and rat), teleost (fish) type and artiodactyl (porcine, bovine and ovine) type. These display considerable differences in amino acid composition, varying from between a difference of two (between rat and human) to 19 (between human and ovine) amino acid substitutions. Eight residues (positions 1, 4, 5, 6, 7, 9, 28 and 32) are common to all species so far studied; of these seven are clustered at the *N*-terminal ring, suggesting

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

* Man2 - predicted

Fig. 2. Amino acid sequences of calcitonins from eight species, including human calcitonin (Man 1 and Man 2*)

Salmon, Sa; chicken, Chick; porcine, Porc.; bovine, Bov.; ovine, Ov. The invariant residues are shown by horizontal lines; two of these have been altered in the predicted (*) second human calcitonin-like sequence (Man 2*). (Reproduced with kind permission from Q. J. Exp. Physiol.)

physiological constraints on this region of the molecule. In the human calcitonin-like peptide predicted from the DNA sequence of the human β gene, two of the eight invariant residues (positions 1 and 9) are altered. The predicted chicken and pigeon calcitonin sequences (Lasmoles *et al.*, 1985) resemble fish calcitonins.

The CGRP sequence consists of 37 amino acids with a disulphide bridge between positions 1 and 7 and a *C*-terminal phenylalanine amide residue (Fig. 3). There is about 30% structural homology between the structures of the CGRP peptides and salmon calcitonin. It is worth noting that the structure of the rat α - and β -CGRP and human β -CGRP have been predicted from the nucleotide sequence (Rosenfeld *et al.*, 1983; Hoppener *et al.*, 1985; Steenbergh *et al.*, 1985), and only in the case of human α -CGRP the primary structure has been determined unequivocally by fast atom bombardment mass spectrometry (Morris *et al.*, 1984). Human α -CGRP differs from the predicted rat sequence by four amino acid substitutions (positions 1, 3, 25 and 35; three of these involve charge changes). Human α -CGRP differs from the β form by three amino acids (positions 3, 22 and 25). Two of the three residues (positions 3 and 25) have also been modified in rat α -CGRP: one of these is replaced with the same amino acid (asparagine). The presence of a methionine in human β -CGRP makes it possible to distinguish it from the α form by treating the

	sCT	h(α)	h(β)	r(α)	r(β)
1	Cys	Ala	Ala	* Ser	* Ser
2	Ser	Cys	Cys	Cys	Cys
3	Asn	Asp	*	*	*
4	Leu	Thr	Thr	Thr	Thr
5	Ser	Ala	Ala	Ala	Ala
6	Thr				
7	Cys				
8	Val				
9	Leu	Thr	Thr	Thr	Thr
10	Gly	His	His	His	His
11	Cys	Arg	Arg	Arg	Arg
12	Leu				
13	Ser	Ala	Ala	Ala	Ala
14	Gln	Gly	Gly	Gly	Gly
15	Glu	Leu	Leu	Leu	Leu
16	Leu				
17	His	Ser	Ser	Ser	Ser
18	Lys	Arg	Arg	Arg	Arg
19		Ser	Ser	Ser	Ser
20		Gly	Gly	Gly	Gly
21		Gly	Gly	Gly	Gly
22		Val	* Met	Val	Val
23		Val	Val	Val	Val
24	Leu	Lys	Lys	Lys	Lys
25	Gln	Asn	* Ser	* Asp	Asn
26	Thr	Asn	Asn	Asn	Asn
27	Tyr	Phe	Phe	Phe	Phe
28	Pro	Val	Val	Val	Val
29	Arg	Pro	Pro	Pro	Pro
30	Thr				
31	Asn				
32	Thr	Val	Val	Val	Val;
33	Gly				
34	Ser				
35	Gly	Lys	Lys	* Glu	* Glu
36	Thr	Ala	Ala	Ala	Ala
37	Pro	Phe	Phe	Phe	Phe
	NH ₂	NH ₂	NH ₂	NH ₂	NH ₂

Fig. 3. The sequences of four CGRP molecules, compared with salmon calcitonin (sCT)

There is approximately 30 % similarity between the amino acid sequences of the CGRP peptides and sCT when a gap of five amino acids is introduced to maximize alignment. The asterisks show the amino acid substitution differences of the CGRP molecules compared with human CGRP (α). Human CGRP α and β, h(α) and h(β); rat CGRP α and β, r(α) and r(β). (Reproduced with kind permission of Q. J. Exp. Physiol.)

peptide with CNBr followed by chromatographic separation (Petermann *et al.*, 1987).

Structure-activity relationships

The highly conserved residues clustered at the two ends of the calcitonin molecule strongly suggest that the termini are important for biological activity. These are thought to be juxtaposed in spatial configuration (Byfield *et al.*, 1972). The variable middle portion of the molecule (position 10–27) controls the potency and duration of action of the peptide. When exposed to antibody-producing cells *in vivo*, this region acts as an immunogen, leading to unwanted antibody production that is known to complicate the treatment of human bone disease with non-human calcitonins.

Ultimobranchial (salmon and eel) calcitonins are more potent than thyroidal calcitonins both *in vivo* and *in vitro* (Galante *et al.*, 1971), due to either a greater affinity for

the receptor (Marx *et al.*, 1972) or greater resistance to metabolic degradation (Habener *et al.*, 1971; deLuise *et al.*, 1972). More recently, it has been suggested that 'conformational flexibility' of the molecule can significantly affect its biological activity (Epand *et al.*, 1986). A more 'flexible' molecule is able to attain a larger number of conformations at the receptor-membrane complex. The flexibility of a molecule has been shown to depend on the bulkiness of its amino acid side chains (glycine is the least bulky) and thus, upon its tendency to form rigid helices (Kaiser & Kezdi, 1984). The identical biological potencies of salmon and eel calcitonin can be explained on basis of similarity between the two peptides in their primary structures and conformational flexibilities. As three glycine residues are highly conserved both in salmon and eel calcitonin, both peptides possess a remarkably low helix-forming potential. This either allows the molecules a more energetically favourable access to the receptor-membrane complex or allows them to escape a less active, though stable, conformation (Epand *et al.*, 1986). Although it appears that the valine residue in eel calcitonin at position 27 might induce conformational rigidity due to its bulky side chain, the effect is effectively cancelled by the presence of an alanine residue at position 29. In addition, replacement substitutions of salmon calcitonin with amino acids having less-bulky side chains (valine with glycine at position 8 or leucine with alanine at position 16) have been shown to produce more potent analogues, again due to an increasingly flexible molecule.

Nevertheless, it is clear that consideration of conformational flexibility is not enough to predict the relative activity of calcitonin analogues; neither is the consideration of any single factor such as hydrophobic moment or overall peptide hydrophobicity. Peptides such as des-Leu¹⁶-salmon calcitonin and human calcitonin have less helix-forming potentials and are, consequently, more flexible than salmon calcitonin, but are much less potent (Epand *et al.*, 1986). In addition, conformational coupling within the various regions of the molecule makes it more difficult to predict the potency of analogues. This is due to long range (with respect to the amino acid sequence) interactions between the *N*- and *C*-regions of the molecules, at least in the receptor-bound form (Pless *et al.*, 1971). It is also possible that the binding of calcitonin to its receptor is coupled to co-operative conformational interactions in different regions of the peptide in a manner analogous to that proposed for the binding of substrate to enzymes (Taniuchi, 1984).

Generally, modifications in the primary structure of calcitonin that cause a reduction or loss of biological activity include deletion of the *C*-terminal proline amide, shortening of the *C*-terminal end, cleavage of the disulphide bond (Rittel *et al.*, 1976) or oxidation of methionine (position 8 in human calcitonin) (Seiber *et al.*, 1970). Those modifications that bring about no change in biological activity include oxidation of the methionine (position 25 in porcine, bovine or ovine calcitonin). Generally, replacement modifications that tend to increase homology with salmon calcitonin have been shown to increase potency (Maier *et al.*, 1974).

Recent work has questioned the importance of the stereochemical arrangement of the *N*-terminal residues produced by the formation of the disulphide bridge. An analogue of salmon calcitonin (des-Ser²-salmon calcitonin) which has fewer amino acids at the *N*-terminus

is fully biologically active (Schwartz *et al.*, 1981), and the replacement of the disulphide bridge in a carba-type analogue of eel calcitonin (eel-asu¹⁻⁷-calcitonin) also results in full retention of biological activity (Morikawa *et al.*, 1976; Zaidi *et al.*, 1987b). Orlowski *et al.* (1987) have demonstrated that the covalent ring structure in salmon calcitonin is not essential for biological activity, having shown that the opening of the disulphide bridge between positions 1 and 7 of salmon calcitonin and its replacement by an *S*-acetamidomethylcysteinyl linkage does not abolish the hypocalcaemic or renal cyclic AMP-stimulating activity of the peptide. Nevertheless, a similar modification in the human calcitonin molecule only leads to the loss of the hypocalcaemic effect and retention only of the renal effect. The authors have, however, stated that in a lipid-bound form, residues 1 and 7 when bound by a disulphide bridge do promote the formation of a helical structure in other regions of the molecule. It has also been clearly demonstrated that an intact hexapeptide ring structure is not essential for the binding of antagonistic arginine-vasopressin analogues to vasopressor or antidiuretic arginine-vasopressin receptors (Manning *et al.*, 1987). These discoveries raise the possibility that other cyclic peptides, such as somatostatin, may also not require a ring structure for activity. Such modifications may form the basis for the design of linear analogues of cyclic peptides as pharmacological probes and for therapeutic use.

Studies on the structure-activity relationship of CGRP have demonstrated that, apart from minor differences, the four CGRP sequences behave similarly (Tippins *et al.*, 1986; Zaidi *et al.*, 1987c). They cause an equivalent elevation in blood flow in the rabbit skin and enhance the contractility of the rat atrium (Tippins *et al.*, 1986; Brain *et al.*, 1986). They also identically inhibit osteoclastic bone resorption (Zaidi *et al.*, 1987c). Using tryptic analogues of CGRP I have found that, as with calcitonin, the intact molecule is necessary for biological activity (Zaidi, 1987). However, unlike salmon calcitonin (Orlowski *et al.*, 1987), we found that linearized carboxy-amido analogue of human α -CGRP was inactive on all systems (Zaidi, 1987). Furthermore, acetylation of either of the lysine residues (positions 24 and 35) or of the *N*-terminus has been shown to cause a significant reduction in biological activity (Zaidi, 1987).

The close structural similarity between salmon calcitonin and CGRP (see above) has been taken to explain the cross-reactivity shared between one peptide and the other's receptor (Tschopp *et al.*, 1985; Goltzman & Mitchell, 1985). It has been suggested that CGRP can attain a similar configuration to salmon calcitonin, due to common size, charge, the presence of a *C*-terminal amidated residue and an *N*-terminal disulphide bridge (Roos *et al.*, 1986). Recent studies on peptide-receptor interactions have emphasized that molecules with relatively little sequence homology can have similar conformational interactions with receptor-membrane complexes (Kaiser & Kezdi, 1984). These questions should be resolved once the structures of the salmon calcitonin receptor and CGRP receptors are known.

BIOLOGICAL ROLE

Receptors and signal transduction

Calcitonin receptors have now been characterized on

isolated osteoclasts. These are the physiological target cells of the hormone. Binding of ¹²⁵I-labelled calcitonin to rat osteoclasts is saturable, highly specific and incompletely reversible. A receptor component having an *M_r* of approx. 80000 has been identified; this is in good agreement with that observed on a breast cancer cell line (Moseley *et al.*, 1982). The density of calcitonin receptors on the osteoclast (10⁶ per cell) is comparable only to that reported for epidermal growth factor receptors on epidermal carcinoma cells (Wrann & Fox, 1979). Previously, high-affinity sites for calcitonin had been demonstrated on whole bone sections (Warshawsky *et al.*, 1980), membrane preparations (Marx *et al.*, 1972) and on 'osteoclast rich' cultures (Luben *et al.*, 1976).

The biochemical events that follow calcitonin receptor activation remain completely unclear. Phenomena such as receptor loss (Tashjian *et al.*, 1978; Findlay *et al.*, 1981b, 1984), receptor down-regulation (Roos *et al.*, 1986), homologous desensitization (Findlay *et al.*, 1981b, 1984) and persistent adenylate cyclase activation (Lamp *et al.*, 1981; Michelangeli *et al.*, 1983) also require further elucidation. The only available reports suggest that calcitonin causes dose-dependent elevations of cyclic AMP levels (Nicholson *et al.*, 1986; Ito *et al.*, 1985) and that dibutyryl cyclic AMP mimics the effect of calcitonin on isolated osteoclasts (Chambers & Moore, 1983). We feel on basis of pilot studies that cyclic AMP may not be the only second messenger involved in the calcitonin effect, and that other, physiologically relevant and interesting possibilities exist (M. Zaidi and others, unpublished work). These require further investigation.

Amongst the more extensively studied non-bone cells, calcitonin receptors have been identified on those of the kidney (Marx *et al.*, 1972, 1973; Goldring *et al.*, 1978), brain (Tschopp *et al.*, 1985; Rizzo & Goltzman, 1981), testes (Chausmer *et al.*, 1982), pig lung (Fouchereau-Peron *et al.*, 1981a), fish gill (Fouchereau-Peron *et al.*, 1981b), lymphoid cell lines (Marx *et al.*, 1974; Moran *et al.*, 1978) and human cancer cell lines (Martin *et al.*, 1981; Findlay *et al.*, 1980a,b, 1982), namely lung cancer (BEN; Hunt *et al.*, 1977; Findlay *et al.*, 1980a) and breast cancer (MCF7 and T₄₇D; Martin *et al.*, 1981; Moseley *et al.*, 1982, 1983; Lamp *et al.*, 1981; Findlay *et al.*, 1980b, 1981a, 1982; Ng *et al.*, 1983). The number of receptors have been found to range from 5000 to 30000 per cell. The functional significance of 'ectopic' or 'inappropriately expressed' receptors for calcitonin on tumour cells is unclear. In tumours associated with elevated calcitonin levels (for review; Martin *et al.*, 1981), these may trap circulating peptide, and perhaps, alter tumour behaviour. Such cancers may thus be subject to calcitonin treatment (Martin *et al.*, 1981). In particular, it has been suggested that the effect of calcitonin in hypercalcaemia associated with lung and breast cancers may at least, in part, be due to the effect of the peptide on the production of osteoclast-stimulating factors from tumour cells. There is also no obvious explanation for the occurrence of calcitonin receptors (Findlay *et al.*, 1980a) on calcitonin-producing lung cancer (BEN) cells (Zajac *et al.*, 1985). It would, however, be interesting to know whether the calcitonin-like product can act on the producer cell to modify its growth or function (an autocrine effect) (Ng *et al.*, 1983).

In the nervous system there appears to be a family of receptors for CGRP and calcitonin. These have been extensively mapped throughout the nervous systems of

both man and rat, using both biochemical and autoradiographic methods (Goltzman & Mitchell, 1985; Skofitsch & Jacobowitz, 1985; Tschopp *et al.*, 1985; Wimalawansa *et al.*, 1987). The highest number of high affinity CGRP-specific binding sites have been found in the cerebellum (molecular and Purkinje layers) and spinal cord (mainly substantia gelatinosa), in addition to other brain regions, including vessels and pia mater. The binding of labelled CGRP is reversible, saturable, time- and temperature-dependent. There is also a considerable overlap between the distribution of CGRP-immunoreactive structures and binding sites (Tschopp *et al.*, 1985), suggesting a widespread involvement of CGRP in a variety of brain functions. A second class of binding sites found in the central nervous system are those that are specific for salmon, but not for human, calcitonin or for CGRP. These have been particularly found in the dorsomedial and anterior hypothalamus. In contrast, high CGRP binding has been found to be restricted to sites that lack receptors for salmon calcitonin (cerebellum, medial geniculate body, mammillary body and lateral hypothalamic and vestibular nuclei). Both peptides have, however, been found to cross-react at the others receptor, and 1000-fold higher (non-physiological) concentrations (Goltzman & Mitchell, 1985). The relative ability of CGRP and human calcitonin to act on the salmon calcitonin receptor may suggest that CGRP could serve as an endogenous ligand for the salmon calcitonin receptor in the central nervous system (Goltzman & Mitchell, 1985). In contrast to calcitonin receptors found in bone, receptors of the central nervous system are not linked to adenylate cyclase. CGRP can, however, stimulate adenylate cyclase activity by acting at the calcitonin receptor in more than one tissue (Wohlwend *et al.*, 1985; Zaidi *et al.*, 1988a).

Highly specific CGRP receptors have been identified both in the intima and media of vessels, particularly in the coronary superior mesenteric, femoral, distal limb and other visceral arteries (Sigrist *et al.*, 1986; Kubota *et al.*, 1985; Wimalawansa *et al.*, 1987) and the atria and ventricles of the heart (Sigrist *et al.*, 1986). Unlike neural receptors, cardiovascular receptors show no cross-reactivity with salmon calcitonin. These receptors are linked to cyclic AMP, and there is a marked stimulation of cyclic AMP accumulation when cultured rat aortic smooth muscle or human or bovine endothelial cells are incubated with CGRP (Kubota *et al.*, 1985). Seifert *et al.* (1985) have demonstrated the presence of a novel specific receptor for CGRP in the acinar cells of the pancreas. Interaction of CGRP with this receptor causes the release of amylase. Physiologically, the peptide may be released into the insuloacinar circulation from the islets of Langerhans, to stimulate enzyme secretion via a cyclic AMP-mediated system. Specific CGRP binding has also been demonstrated on cloned lung and bone cancer cells (BEN and UMR.106 cell lines); these cells also produce immunoreactive CGRP (Zaidi *et al.*, 1987d, 1988b).

Secretion

A C-cell origin for plasma calcitonin has been established for more than two decades (Foster *et al.*, 1964): the peptide is co-secreted in equimolar concentrations with its C-terminal flanking peptide, katalcalcin, from same granules in C-cells (for review: MacIntyre, 1988; MacIntyre *et al.*, 1987a; Zaidi *et al.*,

1987a). CGRP is also present in C-cells of normal thyroid tissue, although it is most evident in tumour cells in medullary thyroid carcinoma, where it is frequently co-stored with calcitonin (Sikri *et al.*, 1985) (for detailed review on the distribution: Zaidi *et al.*, 1987a). The common N-terminal peptide of separate CGRP-containing tumour cells also occurs in medullary thyroid carcinoma, which show no immunoreactivity to calcitonin antisera. The common N-terminal precursor to both peptides does not circulate in intact form in human subjects, but is processed and cleaved before secretion. It is not known how the two precursors or their cleavage products are packaged in the granules of Golgi. It seems likely that only some of the processing has completed before packaging in secretion granules, and that final cleavage steps occur within granules, before secretion (MacIntyre *et al.*, 1987b). It is uncertain whether calcitonin is all secreted in monomer form or whether a significant proportion is secreted as a calcitonin-katalcalcin complex to be processed peripherally. Like other peptide hormones plasma calcitonin immunoreactivity is heterogeneous (for review: MacIntyre, 1984; MacIntyre *et al.*, 1987b), and by analogy with parathyroid hormone, it would not be surprising if a mixture of different forms of calcitonin were secreted from the C-cells. We are also not certain which form(s) of the circulating peptide is (are) biologically active. A detailed characterization of the calcitonin-like immunoreactive material using ultrasensitive osteoclast-based biological assays (Zaidi *et al.*, 1988c,d) will, perhaps, answer such questions.

The early work which demonstrated the release of calcitonin when plasma calcium was elevated (Copp & Cheney, 1962; Kumar *et al.*, 1963) is still the best evidence for the control of its secretion by plasma calcium. Plasma calcitonin levels increase when calcium rises and decrease when it falls. But it is evident that there are other factors. Receptors for 1,25-dihydroxyvitamin D₃ have been identified on C-cells (Freake & MacIntyre, 1982), and it is possible that the hormonal form of vitamin D may have a direct effect on calcitonin secretion. Furthermore, chronic oestrogen administration to women after the menopause elevates plasma calcitonin levels (Stevenson *et al.*, 1981), but it is not clear whether this is a direct or indirect effect. There is also evidence that gastrin and cholecystokinin induce calcitonin secretion (Care, 1970; Cooper *et al.*, 1971). It has been postulated that calcitonin has a special role after eating: its secretion, induced by gastrointestinal peptides, results in greater skeletal retention of calcium than would normally occur.

Studies in experimental animals have demonstrated that circulating CGRP is mainly derived from perivascular and cardiac nerves, when the peptide is released to promote vasodilation (see below) (Zaidi *et al.*, 1985, 1986; Diez Guerra *et al.*, 1988; Emson *et al.*, 1988). The peptide is also secreted with calcitonin from malignant C-cells both in man and in the rat (Zaidi *et al.*, 1986), through the plasma levels of the two peptides are widely discordant. It has been recently demonstrated that in cloned human medullary thyroid carcinoma (TT) cells, a calcium-ionophore, ionomycin, causes a 17-fold increase in the secretion of calcitonin and CGRP (Haller-Brem *et al.*, 1987). It appears, therefore, that the secretion of calcitonin and CGRP from C-cells is, at least in part, modulated by changes in intracellular calcium levels (Haller-Brem *et al.*, 1987).

Actions of calcitonin

The main action of calcitonin, and certainly the one of major physiological importance, is on the bone. When administered acutely into experimental animals with a high bone turnover, or into human subjects with Paget's disease of bone, there is a marked fall of plasma calcium (Robinson *et al.*, 1967; MacIntyre *et al.*, 1987a). The basis of the calcium-lowering effect of calcitonin is an acute inhibitory effect on the osteoclast. Plasma calcium falls, because the inhibition of osteoclastic activity markedly diminishes the flow of calcium from bone to blood. Previous studies have clearly established the inhibitory effect of calcitonin on both normal and stimulated resorption of intact bone (Milhaud *et al.*, 1965; Friedman & Raisz, 1965; Robinson *et al.*, 1967; Aliopoulos *et al.*, 1966; Reynolds *et al.*, 1968). There has also been direct evidence for increased calcium retention in isolated cat tibiae perfused with calcitonin (MacIntyre *et al.*, 1967). In addition, urinary hydroxyproline (an index of bone resorption) has been found to be markedly reduced following calcitonin administration (Martin *et al.*, 1966; Aer, 1968). It has also been demonstrated that osteoclasts (the cells that resorb bone) rapidly lose ruffled borders when either normal or PTH-pretreated bones are incubated with calcitonin (Kallio *et al.*, 1972; Holtrop *et al.*, 1974).

A direct action of calcitonin on the osteoclast had, thus, been deduced soon after the discovery of the hormone (Robinson *et al.*, 1967). The first conclusive demonstration came from Chambers and colleagues, who were able to visualize directly an acute effect of calcitonin on the osteoclast (Chambers, 1982, 1985). Two systems have been used to establish this effect. The first has been the demonstration that calcitonin directly inhibits osteoclastic motility (Chambers & Moore, 1983; Chambers & Ali, 1983; Zaidi *et al.*, 1988c) and spreading (Zaidi *et al.*, 1988c). This has been coupled with the demonstration that calcitonin markedly diminishes the cavities excavated in bone by osteoclastic activity (Chambers & Magnus, 1982; Chambers *et al.*, 1984, 1985a,b; Zaidi *et al.*, 1987c,e, 1988a,c,d). More recently we have developed kinetic techniques to accurately quantify osteoclastic motility. These depend on time-lapse image capture and video recording of a motile osteoclast and computer-assisted mathematical analysis of the digitized images. Motility of osteoclasts is expressed by a simple geometric transformation as described previously by Dunn & Brown (1986). Calcitonin acutely inhibits all components of motility.

Besides the acute effect of calcitonin there are some less well understood effects of the peptide on bone. When calcitonin is administered over several months to patients with Paget's disease, osteoclast numbers have been found to gradually decline (MacIntyre *et al.*, 1987b). Moreover, osteocytes are known to shrink in response to calcitonin. In certain submammalian vertebrate species without a bony skeleton, such as the shark, calcitonin has a unique hypercalcaemic effect (Glowacki *et al.*, 1985). This effect is thought to be mediated via calcium-transporting organs (kidney, intestine or gill). Calcitonin also relieves pain associated with bone disease, by a central analgesic effect (Pecile *et al.*, 1975; Allan, 1983; Frioli *et al.*, 1982). There are many views on the mechanism of calcitonin-induced analgesia: these include effects of the peptide on serotonergic midbrain nuclei (Nakhla & Majumdar, 1978; Clementi *et al.*, 1984, 1985), opiate receptors

(Braga *et al.*, 1978) and the prostaglandin-thromboxane system (Caserani *et al.*, 1979).

Calcitonin also acts on the kidney to enhance the production of 1,25-dihydroxyvitamin D₃ (Rasmussen *et al.*, 1972; Hass *et al.*, 1971). This might represent a true physiological effect of importance during pregnancy and childhood. The effects of PTH and calcitonin are exerted at different sites on the proximal straight tubule (Kawashima *et al.*, 1981): the calcitonin-sensitive site lacks cyclic AMP (Kawashima *et al.*, 1980; Horiuchi *et al.*, 1979; Zalups & Knox, 1983). This effect of calcitonin is probably mediated by prostaglandin E₂ (Yamada *et al.*, 1985). The other effects of calcitonin on the kidney are probably not of physiological importance, although they may be clinically relevant (review: Agus *et al.*, 1981). At the distal nephron, the peptide acts by a cyclic AMP-dependent mechanism (Marx *et al.*, 1972; Heersche *et al.*, 1974; Elalouf *et al.*, 1983), leading to naturesis and diuresis. More recently, it has been shown that calcitonin stimulates cyclic AMP-dependent protein kinase activity and plasminogen activator production and inhibits cell multiplication in a cloned renal tubular cell line, LLC-PK1 (Goldring *et al.*, 1978).

Actions of CGRP

To date there is direct evidence of a role for CGRP in blood flow regulation and its neurotropic effect on striated muscle. The other functions of the peptide have been inferred from its distribution. When administered centrally (intraventricularly or microinjected into the central amygdaloid nucleus), CGRP selectively stimulates noradrenergic outflow; this is accompanied with intense tachycardia and hypertension (Fisher *et al.*, 1983; Nguyen *et al.*, 1986). Paradoxically, when administered systemically CGRP causes hypotension in several species, including humans (Fisher *et al.*, 1983; Lundberg *et al.*, 1985; Tippins *et al.*, 1984; Struthers *et al.*, 1986; Gennari & Fischer, 1986). In man, such effects, accompanied with flushing and catecholamine release, have been noted at plasma CGRP concentrations as low as 56 pmol/l (Struthers *et al.*, 1986): CGRP is probably the most potent direct-acting vasodilator known (Struthers *et al.*, 1986). The hypotensive effects of systemically administered CGRP are due to the direct action of the peptide on blood vessels and not secondary to the release of neurotransmitters: the demonstration of such an effect (Brain *et al.*, 1985), has been the first recognition of the vascular actions of CGRP. Administration of femtomole doses of CGRP intradermally in the rabbit or topically to a hamster cheek-pouch preparation induces intense microvascular (mainly arteriolar) dilatation with increased blood flow. When injected intradermally into human volunteers, the peptide produces reddening or a persistent flare reaction: in this respect CGRP is similar in potency to prostaglandin E₂, and is several-fold more potent than acetylcholine, ATP, ADP, adenosine, 5-hydroxytryptamine, substance P, isoprenaline or vasoactive intestinal peptide (Williams, 1982; Brain *et al.*, 1985).

CGRP dilates a variety of vessels including the coronary, cerebral and systemic vasculature. In man, it has been shown that a dramatic decline of perfusion pressure is coupled with radiographic evidence of dilated coronary arteries (McEwan *et al.*, 1986). Similarly, dilatation has also been demonstrated using the coronary arteries of isolated rat, rabbit and pig heart preparations

(Holman *et al.*, 1986; Al Karwini *et al.*, 1988). Surprisingly, the potencies of rat and human CGRP peptides differ in this respect. The peptide also dilates mesenteric vasculature (Al Karwini *et al.*, 1988) and is a more potent dilator of cerebral arteries than is substance P. The latter effect is more marked *in situ* than *in vitro*, suggesting that CGRP may have a physiological role in preventing cerebral ischaemia due to excessive vasoconstriction (McCulloch *et al.*, 1986). Though it has been established that CGRP is constantly released from perivascular and cardiac nerves, possibly in response to vasosensory stimuli (Zaidi *et al.*, 1985; Diez Guerra *et al.*, 1988; Emson *et al.*, 1988), we are not certain whether the peptide has a true hormonal role in blood flow regulation. It appears that plasma levels merely represent a spill-over phenomenon when CGRP is released from nerve terminals to promote vasodilatation.

The mechanism of CGRP-induced vasodilatation is unclear. The phenomenon appears to be endothelial-dependent in, at least, major vessels (Brain *et al.*, 1985; Kubota *et al.*, 1985). There is some recent unpublished evidence that endothelium-derived relaxing factor (EDRF) may be involved, but whether the same applies to arteriolar dilatation induced by topical application is open to question. In addition, CGRP receptors linked to cyclic AMP have been identified in the red pulp of the spleen (Sigrist *et al.*, 1986) and the peptide has been shown to relax splenic strips precontracted with noradrenaline. It has, however, not been established whether CGRP causes splenic relaxation and filling *in vivo*.

CGRP does not cause protein exudation from microvessels (Gamse & Saria, 1985; Brain & Williams, 1985). Its synergism with histamine, bradykinin, leukotrienes, complement (C5a), platelet activating factor and tachykinins (substance P, neurokinin A and B) in potentiating protein extravasation (Brain & Williams, 1985) is a consequence of arteriolar dilatation. This results in the passive distension of venules that have been acted upon by the aforementioned mediators (Williams, 1982; Brain & Williams, 1985). Indeed, CGRP co-exists with the tachykinins in single cells of sensory neurons (Lee *et al.*, 1985): it seems likely that the peptides may interact to produce neurogenic inflammation or an axon-reflex flare reaction (Foreman *et al.*, 1983; Lembeck & Gamse, 1982; Brain & Williams, 1985). Finally, CGRP increases the rate and force of contraction of the heart muscle in various species via an action on highly specific receptors linked to cyclic AMP (Tippins *et al.*, 1984; Sigrist *et al.*, 1986; Holman *et al.*, 1986; Gennari & Fischer, 1986). In this respect the α form is more potent than β -CGRP. It appears likely that *in vivo*, CGRP, released locally from cardiac nerves, binds to specific functional receptors of the heart.

The significance of CGRP in peripheral visceral and somatic motor relay is evident from its distribution in motor neurons of the ventral spinal cord (Gibson *et al.*, 1984), motor nuclei of the cranial nerves and the nucleus ambiguus (caudal part) (Takami *et al.*, 1985a). CGRP is localized to vesicles (diameter 40–60 nm) in axon terminals, within the synaptic trough of the neuromuscular junction (Takami *et al.*, 1985b) of the striated muscle of the tongue (with acetylcholine) (Lee *et al.*, 1985) and oesophagus (Rodrigo *et al.*, 1985). The peptide enhances diaphragmatic contraction, presumably via its own receptor, and at high doses has a

non-specific inhibitory action on smooth muscle cells of the vas deferens. In addition, CGRP acts on a presynaptic receptor, to inhibit noradrenaline release (Ohhashi & Jacobowitz, 1985). CGRP appears to be the only peptide that has a neurotransmitter role in cardioacceleration, via non-adrenergic, non-cholinergic (NANC) nerves to the sinus node. This response is due to its specific action on pacemaker cells.

More recently it has been demonstrated that CGRP increases the number of acetylcholine receptors on the surface of cultured myotubules (New & Mudge, 1986; Fontaine *et al.*, 1986). The functional significance of this finding is still unclear, but may reflect another novel role for the peptide. The effect appears to be mediated by cyclic AMP, as CGRP enhances the accumulation of cyclic AMP in mouse diaphragm and cultured chick myotubules (Lauffer & Changeaux, 1987; Takami *et al.*, 1986). The effect is also dose-dependent, is observable at physiological concentrations of CGRP and the time course is similar to that of dibutyryl cyclic AMP. Indeed, cyclic AMP has long been suspected to play a role in the development of functional motor end plates (Lentz, 1972). It is unclear whether the potencies of the α and β forms of the peptide are different.

The abundance of CGRP in the central nervous system (Kawai *et al.*, 1985) points towards a neurotransmitter or a neuromodulator role, particularly in the central regulation of visceral autonomic (cardiovascular, respiratory, taste and sleep) and limbic functions (for detailed review: Zaidi *et al.*, 1987a). In several species, including man, CGRP shares with calcitonin the property of producing profound effects on physiological, behavioural and metabolic functions. The basic mechanism of centrally administered CGRP or calcitonin appears to be the depression of neuronal excitability (Twery & Moss, 1985). In particular, it appears that CGRP is a central mediator of ingestive behaviour. As with calcitonin, the peptide suppresses feeding (Krahn *et al.*, 1984), either via an action on the satiety centre or by inhibiting basal and stimulated gastric acid secretion (Lenz *et al.*, 1985). CGRP also probably plays a role in the modulation of involuntary movements mediated by the basal ganglia; its significance in the modulation of auditory responses is, however, unclear (Kawai *et al.*, 1985). The distribution of CGRP in the spinal cord, particularly in laminae containing neurones, responsive to noxious and innocuous stimuli and in ganglia giving rise to C and A (δ) afferent fibres (Gibson *et al.*, 1984), its co-localization with the tachykinins and its depletion following capsaicin (Zaidi *et al.*, 1985; Diez Guerra *et al.*, 1988; Emson *et al.*, 1988), suggest that CGRP may modulate or transmit sensory impulses. Its role as an autonomic neurotransmitter has been suggested on basis of its occurrence in the autonomic ganglia and the diffuse CGRP-rich innervation of the cardiovascular, gastrointestinal, urogenital and special sensory systems (for review: see Zaidi *et al.*, 1987a).

CGRP also has some other effects of pharmacological importance: these are probably exerted via the calcitonin receptor. In mice intravenous infusion causes a sharp rise of renal cyclic AMP levels; the peptide is 10-fold less potent than calcitonin in this respect (Zaidi *et al.*, 1988a). The peptide also binds to the calcitonin receptor on the cloned LLC-PK1 cell line (see above) (Wohlwend *et al.*, 1985). On bone, CGRP shares the acute effects of calcitonin at a 1000-fold higher molar concentration.

For example, the peptide causes hypocalcaemia (Tippins *et al.*, 1984; Roos *et al.*, 1986; Zaidi *et al.*, 1988a), stimulates cyclic AMP formation in mouse calvaria (Zaidi *et al.*, 1988a), inhibits both basal and stimulated resorption of intact bone (PTH-, vitamin D- and prostaglandin-induced) (D'Souza *et al.*, 1986; Roos *et al.*, 1986) and inhibits spreading, motility as well as resorption of bone by isolated osteoclasts (Zaidi *et al.*, 1987c, 1987e, 1988a). The effects *in vivo* of CGRP in the rabbit and chicken are different and partly resemble those of PTH (Tippins *et al.*, 1984). Though in the rabbit the peptide causes hypocalcaemia at doses comparable to those of calcitonin, at higher molar concentrations a sustained PTH-like response follows initial hypocalcaemia. In chicken, CGRP has a PTH-like effect at all doses. In man, systemic CGRP infusion has no observable effect on plasma calcium levels (Struthers *et al.*, 1986). Nevertheless the peptide has been shown to cause a 40–50-fold rise of cyclic AMP levels in normal human osteoblasts (Crawford *et al.*, 1988). The presence of receptors for CGRP on osteoblasts may represent a second and more important effect of CGRP on bone cells. Perhaps a predominantly osteoblastic effect might account for the PTH-like response seen in rabbits and chickens.

CONCLUSIONS AND POSSIBLE FUTURE DIRECTIONS

The study of the biology and genetics of the calcium-regulating hormone, calcitonin, has led to the discovery not only of an unsuspected functional peptide, CGRP, but has also unravelled new systems of regulation. The next decade should see progress in a number of areas.

The factors regulating alternative splicing of the α -calcitonin/CGRP gene are for practical purpose unknown. Given that these factors act only on pre-mRNA, their elucidation appears to be formidable and progress is thus likely to be slow. On the other hand, attempts at elucidating promoter sequences and characterizing factors that regulate gene transcription are more likely to be successful, because techniques used to study the binding of factors to specific DNA sequences and to purify them are comparatively simple. The sequence elements in the promoter region of a gene that affects its transcription can be identified by their binding to specific regulatory factors. The method of analysis is simple: DNA sequences which have bound a protein are protected against DNAase digestion (Gales & Schmitz, 1978) or retarded on gel electrophoresis (Fried & Crothers, 1981). Once the recognition sequence has been identified, the transcription factor of interest can be purified by affinity chromatography using synthetic oligodeoxyribonucleotides of the DNA sequence as binding elements (Kadonga & Tjian, 1986). Indeed, a number of transcription factors have recently been purified using this approach: in one case (AP-1) the factor has been shown to be identical to an oncogene (*c-jun*) (Bohman *et al.*, 1987). These studies would become much easier if cell lines were to become easily available: these could be used to study the expression of *in vitro* mutagenized promoters of genes, thus allowing genetic mapping of regions of interest.

The characterization of receptors for calcitonin and CGRP has been in progress for several years. The scarcity of the starting material and the difficulty in purifying the

active form of the membrane protein because of its hydrophobic domains are major problems. An alternative strategy could be to clone the gene encoding the receptor molecule. Complementary DNA expression libraries could, in theory, be screened with CGRP, the true ligand, but anti-idiotypic immunological screening might be more efficient. Anti-idiotypic antibodies (raised against 'receptor-like' idiotypic determinants of antibodies that have previously been raised against the ligand molecule) have previously been used to identify receptors. The chances of identifying the gene for the receptor would be greatly increased by using the cDNA screening system developed by Seed (Seed, 1987; Seed & Aruffo, 1987). This allows the direct cloning of a full length cDNA in a form containing all the necessary sequences for surface expression, thus obviating multiple rounds of screening to identify full length or overlapping clones. Briefly, it is a technique based on transient expression in *cos* cells and physical selection of expressing cells by adhesion to antibody-coated dishes (so called 'panning'). It allows a large numbers of cDNAs expressing surface molecules to be cloned quickly and allows monoclonal antibodies to be used effectively, unlike screening methods based on fusion protein expression (e.g. λ GT11) and also requires less antibody. In addition, studies on post-receptor signalling in hormone-responsive cells are already underway in our and other laboratories. Photolabile inert (or 'caged') precursor molecules of putative intermediates in the transduction pathway (Gurney & Lester, 1987; Walker *et al.*, 1987) are being introduced into the cell cytoplasm by microinjection. Following their activation by high energy flash photolysis, the behaviour of highly motile effector cells, such as the osteoclast, is studied by time-lapse video- and computer-assisted image analysis. It appears likely that the next decade will unravel regulatory networks in osteoclasts and other hormone-responsive cells.

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