Side-Chain Lactam-Bridge Conformational Constraints Differentiate the Activities of Salmon and Human Calcitonins and Reveal a New Design Concept for Potent Calcitonin Analogues

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We have recently reported the potent hypocalcemic effects of side-chain lactam-bridged analogues of human calcitonin (hCT) (Kapurniotu, A.; et al. *Eur. J. Biochem.* **1999**, *265*, 606−618). To extend these studies, we have now synthesized a new series of (Asp^{17}, Lys^{21}) and (Asp^{17}, Orn^{21}) side-chain bridged salmon calcitonin (sCT) and hCT analogues. The affinities of these analogues for the human calcitonin receptor, $hCTR_{I1-}$, and for rat-brain membrane receptors were assayed in competitive binding assays, and agonist potencies at the $hCTR_{I1-}$ receptors were assessed, using a cAMP-responsive gene-reporter assay. The bridged sCT analogues had activities similar to sCT itself. In contrast, an (Asp^{17}, Orn^{21}) side-chain bridged hCT analogue, *cyclo(17−21)*-[Nle^{8}, Phe^{12}, Asp^{17}, Orn,^{21} Tyr^{22})-hCT, was 80 and 450 times more active than hCT in the $hCTR_{I1-}$ and rat-brain receptor binding assays, respectively, and was 90 times more potent than hCT and 16 times more potent than sCT in initiating receptor signaling. An uncyclized, isosteric analogue of this peptide was also more potent than hCT, demonstrating that the cyclization constraint and these single-residue substitutions enhance the activities of hCT in an additive fashion. This study demonstrates that the potency-enhancing effects of lactam-bridge constraints at hCT residues 17−21 are not transferable to sCT. We also show that, in comparison to the hCT analogues, sCT and its analogues are less potent agonists than expected from their $hCTR_{I1-}$ affinities. This suggests that it may be possible to preserve the efficient signal transduction of hCT while introducing additional receptor affinity-enhancing elements from sCT into our potent lactam-bridged hCT analogue, thereby creating new super-potent, hCT-based agonists.

Introduction

Calcitonin is a peptide hormone comprising 32 amino acid residues that is known primarily for its hypocalcemic effect and inhibition of bone resorption. It has been used therapeutically for the treatment of Paget's disease, the hypercalcemia associated with certain types of tumor, and osteoporosis.[1,2] The relief of pain associated with its application to Paget's disease, and the known antinociceptive properties of calcitonin in animal models,[3] are also suggestive of potential new applications as an analgesic.

The primary structures of calcitonins from a number of species are known, and these show variations in amino acid sequence throughout most of their length.[4] Nevertheless, a number of structural features are conserved that appear to be important determinants of receptor recognition and function. These include a highly conserved N-terminal disulfide loop linking cysteine residues in positions 1 and 7, a potential amphiphilic α-helical structure in residues 8 through 22,[5] and a C-terminal amide. In addition, there are several highly conserved amino acid residues throughout the peptide sequence, including Leu^{4}, Ser^{5}, Thr^{6}, Leu^{9}, Gly^{28}, and Pro^{32}. The most potent calcitonins include salmon calcitonin (sCT^{6}) and eel calcitonin, which are about 50 times more potent than human calcitonin (hCT) and other mammalian calcitonins in vivo. As a result, sCT and an eel-derived analogue, elcatonin, are generally used therapeutically in preference to hCT or other mammalian calcitonins, of which porcine calcitonin has also been used.[1] The salmon and eel calcitonins differ in sequence at only three positions, whereas sCT and hCT differ at 17 positions. In amino acid sequence, hCT is closest to the rat calcitonin structure, which is different at only two positions.

In common with other peptide hormones, calcitonin agonist activities are expressed through binding of the peptide ligand to G-protein coupled receptors (GPCRs) that have the seven-transmembrane-domain structure that is characteristic of this receptor class. Calcitonin receptors (CTRs) from several species, including human, rat, guinea pig, mouse, and rabbit have been cloned,[2,7] and sequence homologies indicate that these receptors belong to a subfamily of homologous GPCRs that includes the receptors for parathyroid hormone (PTH), PTH-related peptide, glucagon, glucagon-like peptide,

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growth hormone-releasing factor (GRF), secretin, and vasoactive intestinal peptide.[8,9] Human and rat CTRs are the best-characterized in terms of their ligand interactions, and these receptors are known to be expressed in multiple isoforms as a result of alternative gene splicing. The major isoforms of the human receptor, $hCTR_{I1-}$[10] and $hCTR_{I1+}$,[11] differ in the absence and presence, respectively, of a 16-residue insert in the proposed first intracellular loop region. In the rat, the major CTR isoforms[12,13] are termed C1a and C1b, and these receptors differ in the absence and presence, respectively, of a 37-residue insert in the putative first extracellular loop. The insert-negative isoform of the human receptor, $hCTR_{I1-}$, appears to be the dominant isoform in most tissues, including the central nervous system,[2,7] and corresponds most closely in sequence to the rat C1a receptor. Both hCTR isoforms bind sCT with identical affinities, but the insert-positive $hCTR_{I1+}$ exhibits impaired signal transduction via the adenylyl cyclase and phospholipase C second-messenger systems compared to $hCTR_{I1-}$.[14] A comparative study of the cloned rat receptor isoforms stably expressed in HEK293 cells indicates that sCT and other calcitonin analogues exhibit considerably higher binding affinities and second-messenger signaling potencies at the rat C1a receptor than they do at the rat C1b receptor.[8]

Understanding the structural and conformational features that determine the receptor interactions and biology of a flexible peptide hormone the size of calcitonin presents a particularly difficult challenge in medicinal chemistry. Such peptides exist in multiple conformational states in aqueous solution, and the receptor-bound conformation(s) required for activity may be poorly represented in the unbound form. Furthermore, the direct determination of the structure of any peptide ligand−receptor complex remains, at present, an intractable problem, due to the integral membrane character of the GPCRs for these ligands. In an attempt to address this issue with calcitonin, we have focused on studying the amphiphilic α-helical region in the middle region of the primary structure of this peptide.[5,15,16] In recent years, evidence has emerged that such structures represent important functional elements of the bound conformations of a number of medium-sized peptide ligands for G-protein coupled receptors, including GRF,[17] glucagon,[18] parathyroid hormone,[19,20] and parathyroid hormone-related peptide.[21] In calcitonin, most commonly used hydrophobicity scales indicate that the amphiphilic character of the potential amphiphilic α helix in calcitonin is conserved throughout residues 8−22 of each of the naturally occurring homologues.[22] NMR studies in mixed solvents, such as methanol−water[23] or TFE−water,[24] demonstrate the presence of a central helix in sCT that extends from residue 8 through residue 22, and similar results have been reported for hCT in TFE solutions.[25] The higher biological potency of sCT compared to hCT generally correlates with indications of a higher helix forming propensity in this central region of the salmon peptide.[26,27] However, the importance of conformational flexibility around residue 8 has also been suggested,[28] and potent sCT analogues that have lost the capability of forming a helix-stabilized complex with lipid micelles have been reported.[29,30] Studies of genetically engineered chimeric

hCT Analogues

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                          10        20        30
hCT:  h-CGNLSTCMLG TYTQDFNKFH TFPQTAIGVG AP-amide

H-1:  h-CGNLSTCMLG TYTQDFDKFH KFPQTAIGVG AP-amide

H-2:  h-CGNLSTCMLG TYTQDFDKFH OFPQTAIGVG AP-amide

H-3:  h-CGNLSTCMLG TYTQDFNPFH TFPQTAIGVG AP-amide

H-4:  h-CGNLSTCMLG TYTQDFNPKH TFPQTAIGVG AP-amide

H-5:  h-CGNLSTCMLG TYTQDFNKPH TFPQTAIGVG AP-amide

H-6:  h-CGNLSTCMLG TYTQDFNPGH TFPQTAIGVG AP-amide

H-7:  h-CGNLSTCBLG TFTQDFDKFH OYPQTAIGVG AP-amide

H-8:  h-CGNLSTCBLG TFTQDFNKFH ZYPQTAIGVG AP-amide
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sCT Analogues

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                          10        20        30
sCT:  h-CSNLSTCVLG KLSQELHKLQ TYPRTNTGSG TP-amide

S-1:  h-CSNLSTCVLG KLSQELNKLH BYPRTNTGSG TP-amide

S-2:  h-CSNLSTCVLG KLSQELDKLH KYPRTNTGSG TP-amide

S-3:  h-CSNLSTCVLG KLSQELDKLH OYPRTNTGSG TP-amide

S-4:  h-CSNLSTCVLG KLSQELDKLQ OYPRTNTGSG TP-amide
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Figure 1. Structures of the synthetic peptides in this study. The single-letter code for each amino acid residue is used. In addition, nonstandard residues are specified by O = ornithine, B = norleucine, and Z = norvaline. Side-chain lactam bridges are indicated by solid lines above the linked residues (|‾‾|).

receptors in which the extracellular, N-terminal domains of the cloned, human glucagon, and calcitonin receptors were swapped have also been very informative.[31] These studies showed that binding of the central region of sCT to the extracellular N-terminal domain of the CT receptor is an important affinity determinant of this ligand but that interactions of the N-terminal disulfide loop in sCT residues 1−7 with the transmembrane and extracellular loop domains of the CT receptor are required for signal transduction. These results are consistent with the antagonist activities of N-terminal truncated sCT analogues, such as sCT(8−32).

To investigate the functional importance of the proposed amphiphilic α-helical structure in the calcitonins, we have adopted the approach of conformationally constraining the central region of hCT, using side-chain lactam bridges.[15] By linking pairs of lysine and aspartic acid residues spaced four residues apart in the linear amino acid sequence, we hoped to stabilize the α-helical conformation in hCT and observe a corresponding change in both receptor binding affinities and pharmacological potencies. To our surprise, we observed that *cyclo*(17−21)-[Asp[17], Lys[21]]-hCT (H-1, Figure 1), which incorporated a lactam linkage that had previously been found to favor α-helix formation,[17,32] was less helical than hCT in a 1:1 (v/v) TFE−water mixture but had a higher affinity for ^{125}I-sCT-labeled sites in rat-brain membranes and was more potent in a hypocalcemic assay in mice.[15] Furthermore, in a follow-up study by Kapurniotu and co-workers,[16] an investigation of the ring-size effects on the hypocalcemic activity of this analogue identified a new lactam-bridged analogue that

was 400-fold more potent than hCT in this assay and about 4-fold more potent than sCT. The activity of this new and potent analogue, *cyclo*(17−21)-[Asp[17], Orn[21]]-hCT (H-2, Figure 1), was particularly supportive of our working hypothesis[15] that the amphiphilic α-helical structure in hCT, if it is indeed stabilized by receptor interactions in the ligand−receptor complex, is terminated by a type-I β turn centered around residues 18 and 19, since the Asp-Orn side-chain linkage in H-2 is too short a constraint to be compatible with formation of a regular α-helical structure through these constrained residues. This type-I β turn in hCT residues 17−20 has been identified by NMR studies of hCT in DMSO−water mixtures, but no stable α-helical structure was observed under these conditions.[33]

We wished to extend these earlier studies in order to investigate the potential for developing new therapeutic agents with potent hypocalcemic or analgesic activities based on the receptor interactions of turn-induced calcitonin analogues. We have, therefore, tested the binding activities and functional potencies of our earlier lactam-bridged hCT analogues at the cloned $hCTR_{I1-}$ receptor and have designed and tested several series of new sCT and hCT analogues based on our earlier discoveries. To allow comparisons with prior structure−activity studies of sCT analogues in particular, we have also tested these analogues in radioreceptor binding assays using rat-brain membranes. We have synthesized and studied a new series of (Asp[17], Lys[21]) and (Asp[17], Orn[21]) side-chain bridged sCT analogues, as well as a series of unbridged hCT analogues with turn-inducing Pro and D residue substitutions for residues 18 and 19. The sCT analogues have been prepared in order to determine whether the potency-enhancing effects of Asp-Lys and Asp-Orn side-chain linkages introduced into hCT can be transferred to the more potent sCT structure with the same results. The unbridged hCT analogues were synthesized in order to explore the possibility of increasing the potency of hCT by stabilizing a β-turn centered around residues 18 and 19, without introducing a cyclic constraint. Two additional analogues were designed and synthesized in order to explore the feasibility of preparing a high-affinity hCT analogue that is suitable for radioiodination. The binding affinities of these analogues for $hCTR_{I1-}$ stably expressed in HEK293−Luc cell membranes and for radiolabeled rat-brain membrane receptors have been assayed in competitive binding assays, using ^{125}I-sCT as the radioligand. In addition, agonist potencies at $hCTR_{I1-}$ were assessed in human neuroblastoma SH−SY5Y cells, using a gene-reporter assay for signal trasduction via the adenylyl cyclase pathway in which luciferase expression is induced by cAMP. The results we now report demonstrate that it is possible to design hCT analogues with agonist potencies at the human receptor that are significantly higher than that of sCT.

Results

Peptide Analogue Design. Analogues of sCT (Figure 1) were designed to investigate the effects of substituting the native residues His[17] and Thr[21] by selected cyclic, side-chain lactam conformational constraints that had previously been demonstrated to enhance the potency of hCT.[15,16] To test the effects of substituting the native residues in positions 17 and 21 without side-chain cyclization, [Asn[17], His[20], Nle[21]]-sCT (peptide S-1) was prepared as an isosteric analogue of the lactam-containing peptide S-2. Peptide S-2 incorporates the Asp[17], Lys[21] side-chain lactam linkage from hCT analogue H-1[15] into the sCT structure, and peptide S-3 incorporates the corresponding Asp[17], Orn[21] lactam from the highly potent hCT analogue H-2[16] into sCT. Peptides S-1, S-2, and S-3 also incorporate a His substitution for Gln[20] in order to retain this side-chain functional group from the substituted position 17 of sCT by placing it in the same position that it is found in the human analogues. Finally, peptide S-4 is an analogue of S-3 that tests the importance of this His[20] substitution by retaining the native sCT residue, Gln, in its place.

In the hCT analogue series, peptides H-3, H-4, H-5, and H-6 were designed to test a variety of potential turn-inducing substitutions[34] for Lys[18] and Phe[19]. These residues have been proposed to adopt a type-I β-turn conformation in receptor-bound hCT,[15] following the solution conformation identified for that site by Motta and co-workers.[33] In this series, the goal was to identify turn-inducing substitutions that would reproduce the potency-enhancing effects of the lactam linkages in H-1 and H-2 without the need for a side-chain to side-chain cyclization constraint. Peptide H-3 has a Pro[18] substitution that is expected to favor a type-I β-turn conformation; H-4 has Pro[18] and D-Lys[19] substitutions that might favor a type-II turn and also retain the side-chain orientation of the native Lys[18] in the proposed type-I turn; H-5 has D-Lys[18] and Pro[19] substitutions to favor a type-I′ turn conformation; and H-6 has D-Pro[18] and Gly[19] substitutions, following the characterization of this residue pair as strongly favoring the type-II′ β-turn conformation.[35]

H-7 and H-8 are peptide analogues of hCT that were designed to investigate the development of a radioiodinated analogue of H-3, the most potent analogue of hCT in an in vivo hypocalcemic assay that has been reported to date.[16] Peptide H-7 incorporates the Asp[17], Orn[21] side-chain lactam linkage of H-3 into its structure, in combination with the substitutions of Met[8] by Nle, Tyr[12] by Phe, and Phe[22] by Tyr. The Met[8] substitution by Nle is isosteric and eliminates the possibility of oxidation of this residue during an iodination reaction for radiolabeling. The Tyr-to-Phe and Phe-to-Tyr substitutions move the position of the single Tyr residue in hCT out of the hydrophobic face of the potential amphiphilic α helix, where iodination is expected to interfere with receptor binding, into position 22, where the single Tyr in sCT is found. Iodination of sCT at Tyr[22] is reported to enhance its binding affinities for $hCTR_{I1-}$ and $hCTR_{I1+}$ about 10-fold and to enhance its potency in stimulating cAMP formation *via* $hCTR_{I1-}$ by a factor of 25.[36] Peptide H-8 is an isosteric analogue of H-7, where the lactam linkage in positions 17 and 21 are replaced by unlinked Asn[17] and Nva[21] residues. H-7 tests the accumulative effect of all of the hCT substitutions made to generate H-7 in the absence of the lactam conformational constraint.

Circular Dichroism Spectropolarimetry. CD spectra of all of the peptide analogues have been measured in 10 mM phosphate buffer solution at 25 °C, and in a

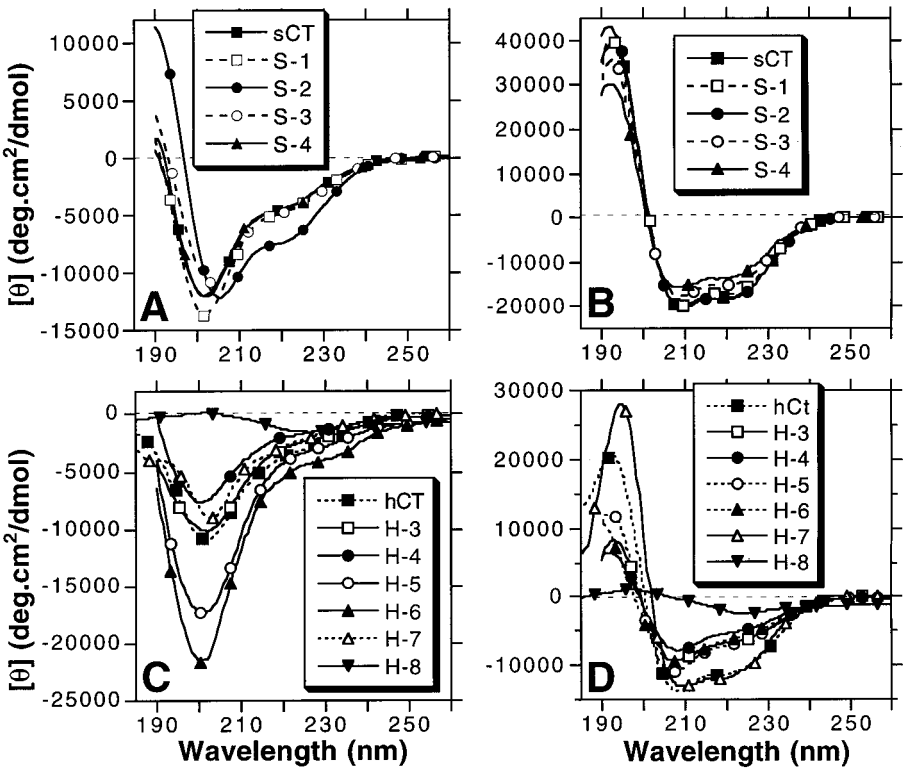

Figure 2. Circular dichroism spectra of peptides. Peptide spectra were measured at 25 °C in aqueous 10 mM NaH_2PO_4−NaOH buffer, pH 7.0 (A and C), or in the same aqueous buffer diluted by 50% (v/v) TFE (B and D). Analogues of sCT are compared to sCT in the same panels (A and B), and analogues of hCT are compared to hCT in the same panels (C and D).

50% (v/v) mixture of this buffer with TFE. Peptide concentrations were in the range 20−50 μM, and 1 mm path length cells were used. Aqueous spectra were also measured at 5−10-fold lower concentrations, using 10 mm cells, without observing significant differences in the spectra. Results for hCT, H-1, and H-2 have been reported previously.[15,16] In aqueous solution, sCT produces a CD spectrum with a strong negative mean residue ellipticity signal, [θ], at around 200 nm, which is indicative of mostly disordered or polyproline-II like structures.[37] In addition, there is a small shoulder in the negative signal at around 220 nm, which is consistent with the presence of some helical structure (Figure 2A). Very similar spectra were produced by aqueous solutions of the unconstrained analogue S-1, as well as the (Asp^{17}, Orn^{21})-bridged analogues S-3 and S-4. Only the (Asp^{17}, Lys^{21})-bridged analogue S-2 is significantly differentiated in the group of sCT analogues, as it gives a strong positive ellipticity peak near 190 nm and a more pronounced negative ellipticity peak at around 220 nm. This is consistent with stabilization of α-helical structure by the Asp, Lys side-chain lactam bridge in this peptide, in the amphiphilic α-helical region. The Asp-Lys side-chain bridge is known to stabilize α-helical structure in peptide hormones and model peptides.[17,32] In contrast, the Asp-Orn bridge in S-3 and S-4 is too short to allow the undistorted propagation of this helical structure from the N-terminal end of the peptide through residues 17−21. Assuming that the $[\theta]_{222}$ signal arises solely from helical structures in these peptides, calculations of the percent helix in these peptides support this analysis (Table 1), indicating 20% helix for S-2 and only 12−13% helix for sCT and S-1, S-3, and S-4.

Table 1. CD Data and Estimated Helix Contents of Calcitonin

	$[\theta]_{222}$ (deg cm²/dmol)		estimated % α-helix[a]	
peptide	aqueous buffer	50% TFE	aqueous buffer	50% TFE
hCT	−3488	−11016	10	30
H-3	−2715	−6506	7	18
H-4	−1834	−5119	5	14
H-5	−3842	−6954	11	19
H-6	−4941	−6043	14	17
H-7	−2600	−11300	7	31
H-8	−1400	−2320	4	6
sCT	−4240	−17811	12	49
S-1	−4476	−16967	12	47
S-2	−7108	−18095	20	50
S-3	−4543	−14867	13	41
S-4	−4200	−13400	12	37

[a] Helix content was estimated based on $[\theta]_{222}$,[38] according to the equation: % helix = $[\theta]_{222}/[\theta]_{max}$, where $[\theta]_{max}$ = −39 000(1 − [2.57/n]) deg cm²/dmol, and n = 32..

The CD spectra of sCT and its analogues in 50% TFE (Figure 2B) are all indicative of an increase in the content of α-helical structure, as expected from the general peptide helix-stabilizing effects of TFE as a cosolvent, and prior NMR studies of sCT in this solvent mixture identifying residues 8−22 as α-helical.[24] In these conditions, both sCT and the unconstrained control peptide, S-1, have a helix content similar to that of the Asp-Lys-bidged S-2 analogue (50%, Table 1), consistent with the propagation of the helix through the entire amphiphilic α-helical region (residues 8−22) in all three peptides. Furthermore, the disrupting effect of the Asp-Orn bridge on the propagation of this helical structure through residues 17−21 is demonstrated by the fact that analogues S-3 and S-4 do not attain as high a helix content in this solvent (about 40%).

In aqueous buffer, hCT has a CD spectrum similar to that of sCT and indicative of a mostly disordered structure with a small amount (about 10%) of α-helical structure (Figure 2C and Table 1). Several hCT analogues, including the pharmacologically more potent Asp-Lys-bridged and Asp-Orn-bridged peptides H-1 and H-2, described previously,[15,16] and the Pro[18]-substituted analogues H-3 and H-4, give CD spectra in aqueous buffer similar to that of hCT itself. The analogues with D-amino acid residues in position 18, H-5 and H-6, both display a more pronounced minimum in the CD spectra at around 200 nm, which may indicate formation of more polyproline-II or disordered structure.[37]

In 50% TFE, the conformational characteristics of hCT and analogues H-1 through H-7 are separated out in a different fashion (Figure 2D). Like the analogues H-1 and H-2 previously reported, hCT and analogues H-3, H-4, H-5, H-6, and H-7 are all more helical in 50% TFE than in buffered water. However, only the Asp-Orn-bridged analogue, H-7, attains a helical content similar to that of hCT itself in this solvent (about 30%), and both hCT and H-7 are notably less helical than sCT in the same conditions (Table 1). We have suggested previously[15] that the Asp-Lys bridge of H-1 destabilizes helical structure in 50% TFE relative to hCT by favoring the proposed type-I β-turn conformation centered around residues 18 and 19. This would have the effect of limiting the extension through these residues of the weakly stabilized α-helical structures that may be forming in the amphiphilic helical region. Likewise, the Asp-Orn bridge in H-2, and the Pro and D-residue substitutions in positions 18 and 19 in H-3, H-4, H-5, and H-6, are also expected to lower the overall TFE-stabilized helix content in this region. Therefore, since H-7 and the previously reported H-2 both incorporate the same Asp-Orn lactam bridge linking residues 17 and 21, the additional substitutions in the H-7 sequence must be compensating for the helix destabilizing effect of this bridge by stabilizing helical structure in other parts of the peptide chain. The most likely candidates for such a helix stabilizing effect are the substitutions of Nle for Met[8] and Phe for Tyr[12], since the remaining substitution is Tyr for Phe[22], which is sandwiched between two helix-terminating structures, the lactam bridge and Pro[23].

Finally, it is noted that analogue H-8, which provides an isosteric control for the effect of the lactam bridge in H-7, produced weakly negative and relatively featureless CD spectra that were distinctly different from those of all of the other analogues in this study, both in aqueous buffer and in 50% TFE. The aqueous spectra were not concentration dependent in the range measured (5−50 μM). Nevertheless, since this peptide was difficult to solubilize, it is possible that these spectra resulted from the formation of an aggregated state, possibly including the formation of fibrils such as hCT itself is prone to form under certain conditions.[39] This phenomenon was not investigated further.

Peptide Affinities for the Human Calcitonin Receptor. Peptide binding to the cloned human calcitonin receptor, $hCTR_{I1-}$, stably expressed in HEK293-Luc cells transfected with an expression plasmid (pcDNA 3.1/Zeo/GC2), was assayed by competitive inhibition of [^{125}I-Tyr[22]]-sCT binding to the washed cell membranes at 25 °C. The receptor assay was characterized by determining saturation binding curves for the radioligand. Curve-fitting of the binding isotherms using the software program LIGAND[40] gave a best fit for a single site model, with a K_D for the radioligand of 45.3 ± 8.8 pM and a receptor population, B_{max}, of 80.1 ± 17.6 fmol/mg protein (n = 5). Specific binding of 50 pM [^{125}I]-sCT was linear with membrane protein concentrations up to 140 μg/mL. A membrane concentration of 40−60 μg protein/tube was used for all subsequent competitive binding assays. GppNHp pretreatment of the cell membranes (100 μM GppNHp in the presence of 100 mM NaCl for 30 min), followed by washing with buffer, induced a 4−5-fold shift in the radioligand affinity without affecting the binding capacities (B_{max}), indicating that the majority of the $hCTR_{I1-}$ receptors that are expressed in this HEK-Luc cell line are correctly folded in the cell membrane and G-protein coupled. In competitive radioligand-displacement studies, a comparison of the affinities of the native peptides sCT, hCT, eel CT, and amylin for the $hCTR_{I1-}$ receptor gave results consistent with previously reported literature values:[2,41] sCT bound with a K_i of 51 ± 9 pM, which was slightly lower than the K_i measured for eel calcitonin and approximately 800 times lower than the K_i measured for hCT. Amylin binding was the weakest of this group.

Table 2. Binding Affinities of Peptides for ^{125}I-sCT-Labeled $HCTR_{I1-}$ Receptors

	$hCTR_{I1-}$ receptor binding			
peptide	K_i (nM)	standard error (nM)	n	relative affinity (%)[a]
hCT	42.5	3.4	3	0.12
H-1	195	41	3	0.026
H-2	not tested			
H-3	1229	226	3	0.0041
H-4	2569	587	3	0.0020
H-5	400	108	3	0.013
H-6	>2500		3	<0.0020
H-7	0.56	0.18	4	9.1
H-8	5.28	1.75	4	0.97
sCT	0.051	0.009	4	100
S-1	0.069	0.017	3	74
S-2	0.050	0.017	3	100
S-3	0.069	0.024	3	74
S-4	0.023	0.006	3	220
eel CT	0.172	0.061	4	30
amylin	164	65	3	3.1

[a] Based on sCT affinity = 100%.

The sCT analogues all bound to $hCTR_{I1-}$ with affinities similar to that of sCT itself, indicating that the lactam-bridge constraints and single-residue substitutions introduced into the sCT structure in positions 17, 20, and 21 had little effect on its binding affinity for this isoform of the human receptor. The Asp-Orn-bridged analogue S-4 had the highest affinity in this group of analogues, but it had only a 2-fold higher affinity than sCT itself.

In contrast to the rat-brain assay results reported previously,[15] constraint of the hCT structure by an Asp[17]-to-Lys[21] side-chain lactam bridge (H-1) actually lowered its affinity for the human receptor somewhat (Table 2). However, a comparison of the K_i values obtained for the isosteric analogues H-7 and H-8 in this assay shows that the Asp-Orn bridge in this position in the hCT sequence increases the binding affinity for

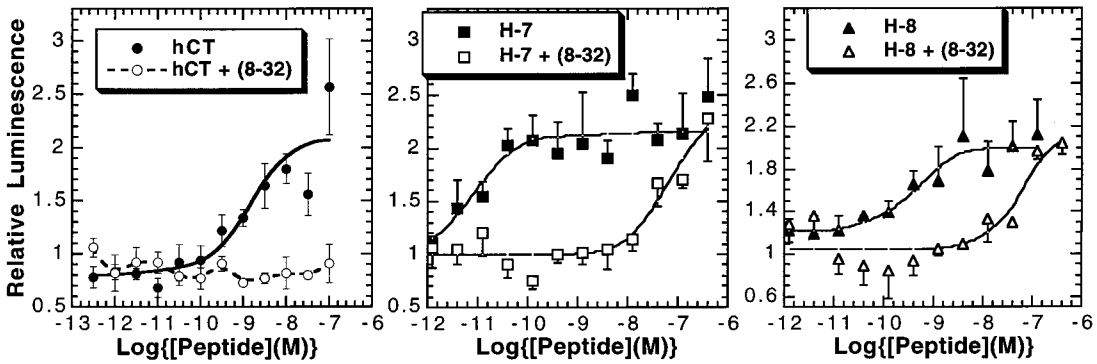

Figure 3. Assays of adenylyl cyclase activation. Peptide activation of the cAMP second-messenger system via binding to $hCTR_{I1-}$ was determined using a CRE-luciferase gene-reporter assay. Data are presented as the luminescence intensities relative to baseline, as a function of the peptide concentration (nM) expressed on a log scale. Co-incubation with the calcitonin antagonist sCT(8−32) (1.0 μM) is indicated as +(8−32) in the figure key. Solid lines indicate the best fit of the data as described in the text, and error bars represent standard errors in the measured increase in luminescence intensities.

$hCTR_{I1-}$ by a factor of about 10. Furthermore, the receptor binding affinity of the unconstrained analogue, H-8, was still 8-fold greater than that of hCT itself, demonstrating that the affinity-enhancing effects of single-residue substitutions made in H-7 relative to hCT, and those of the lactam cyclization, are additive. As a result, H-7 has a K_i for $hCTR_{I1-}$ that is 76 times lower than that of its parent peptide, hCT, and only 11 times higher than that of sCT.

In the $hCTR_{I1-}$ binding assay, the ability of hCT to displace radiolabeled sCT binding was higher than those of the Pro- and D-residue substituted linear analogues, H-3, H-4, H-5, and H-6. In this group of analogues, the lowest K_i value was obtained for peptide H-4 ([D-Lys^{18}, Pro^{19}]-hCT), but this was nearly 10 times higher than that obtained for hCT. These data suggest that the side-chain functional groups in this region of hCT may be important for recognition by $hCTR_{I1-}$ and/or that these residues are not good mimics of the conformational constraint introduced into this region by the Asp-Orn lactam bridge.

Peptide Activation of the Human Calcitonin Receptor. The signal transducing potencies of peptide ligands at $hCTR_{I1-}$ were assessed in SH-SY5Y cells that were transiently cotransfected with plasmids carrying the human receptor gene and a cAMP-responsive MRE/CRE luciferase gene-reporter construct.[42] Forty-eight hours after cell cotransfection, peptides were assayed for their abilities to signal luciferase expression via $hCTR_{I1-}$ activation and coupling to the adenylyl cyclase second-messenger pathway. Peptide activities were assessed after co-incubation with the cells at 37 °C for 4 h, followed by chemiluminescence assay of the intracellular enzyme activity. Active peptides gave sigmoidal log-[peptide] vs response curves that had similar maximal effects at higher concentrations (Figure 3). EC_{50} values were obtained for each peptide by curve-fitting analysis (Table 3). In preliminary control experiments, we verified that sCT failed to produce any luminescent signal in SH-SY5Y cells transfected with the gene-reporter construct only, proving the absence of endogenously expressed receptors functionally interacting with sCT in this cell system. HEK293-Luc cells expressing $hCTR_{I1-}$ were not used for these studies as the parental HEK293 cells showed a response to calcitonin per se. This response was not due to calcitonin receptor activation, as quantitative Taqman assessment of mRNA did not reveal the presence of sequence encoding either the $hCTR_{I1-}$ receptor or the $hCTR_{I1+}$ receptor. Further, there was no specific ^{125}I-sCT binding observed in membranes prepared from these parental cells (data not shown).

Table 3. Potencies of Peptides in the $HCTR_{I1-}$ CRE-Luciferase Assay

	$hCTR_{I1-}$ receptor activity			
peptide	ED_{50} (nM)	standard error (nM)	*n*	relative potency (%)[a]
hCT	0.60	0.1	9	19
H-1	377	148	3	0.031
H-2	not tested			
H-3	99	17	3	0.12
H-4	104	68	2	0.11
H-5	22	4	3	0.52
H-6	>400		2	<0.029
H-7	0.007	0.002	3	1600
H-8	0.27	0.08	3	43
sCT	0.115	0.015	13	100
S-1	0.046	0.010	3	250
S-2	0.075	0.015	3	150
S-3	0.053	0.014	3	220
S-4	0.091	0.043	3	130
amylin	13.5	1.5	2	0.85

The potencies of the native peptides hCT, sCT, and amylin at $hCTR_{I1-}$ in this CRE-luciferase assay corresponded to results reported elsewhere.[43] Compared to sCT, hCT was approximately five times less potent, and amylin was over 100-fold less potent (Table 3). However, the potency of hCT relative to sCT was significantly greater than would be expected based on the relative binding affinities for these two peptides in the $hCTR_{I1-}$ binding assay, where it was over 800 times less active.

A comparison of the potencies of hCT and H-1 in activating receptor signaling shows that the Asp^{17}, Lys^{21} side-chain bridge constraint results in decreased potency relative to hCT. In contrast, analogues H-8 and H-7 show incremental increases in their potencies of factors of 2 and then 40, respectively, as first single-residue substitutions and then the Asp^{17}, Orn^{21} side-chain lactam bridge are introduced. These activities follow the differences in $hCTR_{I1-}$ binding affinities for these ligands that are listed in Table 2. As expected for a direct ligand:receptor interaction, co-incubation with a calcitonin-receptor antagonist peptide, sCT(8−32),[44] retarded the activities of hCT, H-7, and H-8 in this

assay. Agonist concentrations of approximately 100 nM were now required for receptor activation (Figure 3). This provides additional evidence that the potent intracellular signaling activities of these analogues resulted from specific activation of the insert-negative human calcitonin receptor, $hCTR_{I1-}$. Constrained analogue H-7, which exhibits a very high potency in this assay ($ED_{50} = 7 \pm 2$ pM), is 16 times more potent than sCT and 86 times more potent than its parent peptide, hCT.

Our attempts to mimic the proposed β-turn inducing effects of the successful lactam bridge constraints in H-1, H-2, and H-7, by using Pro and D-residue substitutions for hCT residues 18 and 19, were unsuccessful, as judged by the luciferase assay. These results also parallel the results obtained in the $hCTR_{I1-}$ binding assay. Analogues H-3, H-4, H-5, and H-6 were all much less potent than hCT, indicating the sensitivity of the interactions of hCT with this human receptor to changes in these residues. H-5 was again the most potent analogue, but was nearly 40 times less potent than hCT.

All of the sCT analogues were slightly more potent than sCT itself in the CRE-luciferase assay, but only within a narrow range. The most potent analogue, S-2, was only about twice as potent as sCT. These activities are, therefore, consistent with the measured binding affinities of the sCT analogues at the human receptor (Table 2) in showing that the lactam-bridge and single-residue substitutions made at positions 17, 20, and 21 in these analogues do not alter the biological activities of sCT significantly. In particular, the dramatic potency enhancing effect of the Asp^{17}, Orn^{21} side-chain lactam bridge constraint on the potency of hCT in this CRE-luciferase assay was not realized in the context of the sCT sequence (S-3 and S-4).

Peptide Affinities for Rat-Brain Membrane Receptors. Peptide binding to rat-brain calcitonin receptors was assayed by competitive inhibition of [^{125}I-Tyr^{22}]-sCT binding. Results are reported in Table 2 as IC_{50} values and as activities relative to sCT. The use of the radioligand at very low concentrations (10 pM) relative to the reported dissociation constants for sCT binding to rat-brain receptors[8,12] ensures that the IC_{50} values for competitive inhibition by our calcitonin analogues closely reflect their relative K_i values for binding to these receptors.

The relative ordering of the inhibitory activities of all of the hCT and sCT analogues assayed for rat-brain receptor binding generally followed the order found for $hCTR_{I1-}$, binding and signal activation. Consistent with previous reports,[15,45,46] sCT competes effectively with ^{125}I-sCT for binding to rat-brain receptors in the 0.1−1.0 nM range, but hCT binds very poorly to these radiolabeled sites and exhibits an inhibitory activity about 6500 times lower than that of sCT (Table 4 and Figure 4). The Asp-Orn-bridged hCT analogue, H-2, was found to be about 20 times more active than hCT in this binding assay, which is consistent with its higher potency in the hypocalcemic assay in mice.[16] However, despite exhibiting a 2- to 3-fold more potent hypocalcemic effect in vivo than even sCT, it is still about 300 times lower in affinity than sCT in this receptor binding assay. Similar results had previously been reported for the Asp-Lys-bridged hCT analogue, H-1.[15]

Table 4. Binding Affinities of Calcitonin Analogues for ^{125}I-sCT-Labeled Sites in Rat Brain

	rat-brain receptor binding			
peptide	IC_{50} (nM)	standard error (nM)	*n*	relative affinity (%)[a]
hCT	2450	617	4	0.015
H-1	(60)[b]			(0.6)[b]
H-2	173	25.6	3	0.21
H-3	1905	253	3	0.020
H-4	>5000[c]		2	<0.0075
H-5	>5000[c]		2	<0.0075
H-6	>5000[c]		4	<0.0075
H-7	5.39	0.121	4	7.0
I-H-7	21.9	4.21	3	1.7
H-8	79	19.8	3	0.48
sCT	0.377	0.148	4	100
S-1	0.408	0.103	3	92
S-2	0.180	0.032	4	210
S-3	0.249	0.027	5	150
S-4	0.326	0.075	4	120

[a] Based on sCT affinity = 100%. [b] Estimated from data reported previously using similar methods.[15] [c] Less than 30% ^{125}I-sCT displacement at 3.16 μM.

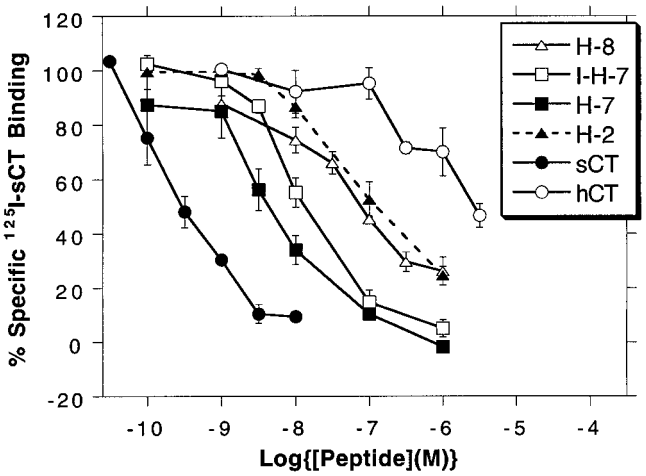

Figure 4. Rat-brain membrane radioreceptor binding assays. Peptide displacement of the percentage of specific ^{125}I-sCT binding is plotted against the competing peptide concentration (nM) on a log scale. Error bars indicate the standard errors in the percentage of specific binding.

A comparison of the rat-brain binding activities of analogues H-1 and H-2 with those of the sCT analogues S-1, S-2, S-3, and S-4 (Table 4) indicates that, in this assay also, transferring the Asp-Lys or Asp-Orn side-chain lactam-bridge constraints into the sCT structure produces much smaller effects on the binding affinity of sCT. As for $hCTR_{I1-}$ binding, all of these sCT analogues gave IC_{50} values in the rat-brain binding assay that were very similar to that of sCT: in this case, the most active analogue in this group was the Asp-Lys-bridged peptide, S-2, but the IC_{50} measured for S-2 was only 2-fold lower than that of sCT itself.

The rat-brain binding affinities of the turn-induced hCT analogues, H-3, H-4, H-5, and H-6, again indicate that the introduction of Pro and D-residue substitutions in positions 18 and 19 is generally detrimental to receptor binding. In this group, only the [Pro^{18}]-hCT analogue, H-3, had an inhibitory activity similar to that of hCT itself. This suggests that elimination of the Lys^{18} side chain is either simply tolerated, because there is no corresponding receptor interaction, or the lost receptor interaction with this side chain is compensated for

by the conformational constraint introduced by the Pro[18] substitution.

In contrast to the Pro- and D-residue-substituted linear hCT analogues, the linear analogue H-8 had a binding affinity about 30-fold higher than that of hCT in the rat-brain assay. H-7 is isosteric to H-8, but conformationally constrained by an Asp-Orn side-chain lactam bridge. This modification resulted in an additional 15-fold increase in affinity. Considering the IC_{50} values obtained for hCT, H-2, H-7, and H-8 together demonstrates that the rat-brain receptor affinity-enhancing effects of the single-residue substitutions in H-7 and the conformational constraint of the Asp[17]-Orn[21] side-chain lactam bridge are additive (Figure 4). Used in combination, these structural modifications produce an hCT analogue (H-7) that is about 500 times more active than hCT and only 15 times less active than sCT in its affinity for the ^{125}I-sCT-labeled rat-brain receptors. Again, these data follow the results obtained for binding and activation of $hCTR_{I1-}$.

Discussion

The hCT and sCT analogues in this study were designed to explore and extend our earlier reports on the effects of conformational constraint of hCT residues 17−21 using side-chain lactam bridges. In these studies, covalently linking the side chains of Asp[17] and Lys[21], or Asp[17] and Orn,[21] or other residue pairs substituted in the same positions[15,16] was found to enhance the potencies of the parent peptide, hCT, up to 400-fold in hypocalcemic assays in mice, 1 h after subcutaneous injection. In our earlier work, the Asp-Lys constrained analogue was also found to have a higher affinity than hCT for ^{125}I-sCT-labeled sites in rat-brain membranes. This suggested that the enhanced hypocalcemic potencies of these constrained analogues might result from their having higher affinities for the calcitonin receptors that signal this effect, rather than (or in addition to) alternative effects on biological potency in vivo, including increased resistance to proteolysis or improved biodistribution. If, indeed, these constrained analogues bind more tightly to calcitonin receptors, this indicates that the constraints introduced into their structures are stabilizing their receptor-bound conformations in solution, prior to the receptor binding step or steps. We have suggested that a type-I β-turn located at residues 17−20 might be the conformation that is induced or mimicked by these lactam-bridge constraints and that may be responsible for their enhanced potencies. A type-I β-turn at this position was the only ordered conformation that was observed in NMR studies of hCT performed in 85% DMSO,[33] and the presence of Asn[17] in the hCT sequence is consistent with stabilization of this conformation in the native peptide.[34]

We wished to explore further the utility of introducing β-turn-inducing conformational constraints into calcitonin residues 17−20 for generating analogues with the potential for therapeutic use as hypocalcemic or analgesic agents. Therefore, we have chosen in this study to focus on the binding affinities and agonist activities of our earlier analogues, as well as several new ones, at the major isoform of the human calcitonin receptor, $hCTR_{I1-}$. We have investigated the conformational and pharmacological effects of transferring lactam bridge constraints that successfully enhanced the pharmacological activities of hCT into the sCT peptide sequence, since sCT itself has a much higher affinity for calcitonin receptors than hCT and is more potent in vivo (analogues S-1 through S-4). We have also extended these studies to include a series of four new hCT analogues (H-3 through H-6), designed for the induction of various turn conformations without cyclization, through the substitution of Pro and/or D-amino acid residues in positions 18 and 19. Finally, we have investigated two additional analogues of hCT, H-7 and H-8, which were designed to explore the potential utility of radioiodinated peptides based on the potent lactam-bridged hCT analogue, H-2, in studies of calcitonin receptor pharmacology. In addition, to facilitate further comparisons with earlier studies of structure−activity relationships in calcitonins, we have also studied the rat-brain receptor binding affinities of these analogues.

CD studies of the sCT analogues in comparison to sCT itself, conducted in aqueous buffer and in 50% TFE indicate clearly that the Asp[17], Lys[21] lactam bridge introduced into S-2 is helix stabilizing in sCT in aqueous solution (Figure 2A). This effect is not a simple result of the substitution made at residue positions 17 and 21, but depends on the side-chain cyclization, since the isosteric analogue S-1 is no more helical in aqueous buffer than sCT itself. The higher helix content observed in S-2 is consistent with earlier studies of, for example, GRF analogues, where helix stabilization was also found to result from an Asp[17], Lys[21] lactam bridge substitution in a peptide sequence known to have a propensity for helix formation.[17] This result, therefore, further emphasizes a difference between the native sequence of sCT compared to hCT in their central regions, since the same substitution in hCT (H-1) has been found to be helix destabilizing.[15] Under helix-stabilizing conditions, such as the 50% TFE solutions tested in this study, NMR studies indicate that the native sCT helix is already fully stabilized and extended through this lactam-bridged region.[24] Therefore, the helix stabilizing effects of the Asp-to-Lys side-chain bridge in S-2 are no longer in evidence compared to sCT and S-1, and all three of these analogues are about 50% helical (Figure 2B and Table 1). In comparison, the (Asp[17], Orn[21])-bridged sCT analogues, S-3 and S-4, show a lower helical content. This indicates that extension of the α-helix from the N-terminal side through this smaller-sized ring constraint is blocked, as expected from an examination of molecular models, which indicate that the Asp-Orn side-chain linkage is too short to be compatible with a regular α-helical structure.

The lower stability of helical structure in the central region of hCT compared to sCT is highlighted by the lower helix content observed for the human peptide in 50% TFE (Figure 2D and Table 1). Differences between the analogues that are based on the human sequence are only revealed in helix stabilizing solvent conditions, since none of these peptides has significant helical structure in aqueous solution. The CD spectra in 50% TFE indicate that the substitution of turn-inducing residues into positions 18 and/or 19 (analogues H-3 through H-6) has effectively reduced the amount of helical structure in these peptides relative to hCT. This is consistent with their design objective, which was to

terminate the potential amphiphilic α helix in hCT residues 8−22 at the induced turn, and follows the earlier reported helix-destabilizing effects in this solvent system of the potency enhancing Asp^{17}, Lys^{21} side-chain bridge in H-1.[15] However, none of these unbridged hCT analogues were found to be more effective than hCT in the assays presently employed.

In contrast to the unbridged hCT analogues, it is most interesting to observe that the (Asp^{17}, Orn^{21})-bridged analogue, H-7, which we now report to have high affinity and agonist activity at the human receptors and a high affinity for rat-brain receptors, has essentially the same helix content in 50% TFE as hCT. The helicities of S-3 and S-4, compared to sCT, S-1, and S-2, indicate that a lower helix content than that of hCT should be observed for H-7 in these solvent conditions because of the helix-terminating effects of the Asp-to-Orn side-chain bridge incorporated into its structure. This suggests that compensating, helix-stabilizing effects are in effect in H-7, presumably affecting residues 8−17. Helix stabilization in this region of H-7 may be a direct result of substitution of hCT residues 8 and 12. Alternatively, a tertiary structure in which the amphiphilic α-helical region in residues 8−17 is folded against the peptide chain on the C-terminal side of the lactam bridge may be involved. The possibility that peptide aggregation is the source of this helix stabilization also cannot be ruled out by our present studies, but we have not observed any concentration dependence to the CD spectrum of peptide H-7 in aqueous buffer in the limited range tested (5−50 μM). Unfortunately, possible helix stabilizing effects of the additional substitutions in the sequence of H-7 cannot be tested independently by examination of the isosteric, unconstrained analogue, H-8, because the CD spectra of this peptide indicate that it adopted an entirely different, ordered conformation that we suspect does result from aggregation.

The $hCTR_{I1-}$ and rat-brain membrane-receptor binding affinities reported in Tables 2 and 4 for hCT, sCT, and the new analogues are extended over 3 and 4 orders of magnitude for the human and rat receptor assays, respectively. This wide range of receptor binding affinities allows an accurate comparison of the structure−activity relationships obtained from these two assays. A log−log plot of the IC_{50} values from the rat-brain receptor assay against the K_i values from the human receptor assay (Figure 5) indicates a strong linear correlation between these two assays for all of the analogues tested. This suggests that the rat-brain receptor assay is strongly predictive of binding affinities at $hCTR_{I1-}$, and that similar conformational and recognition requirements for these ligands are in operation at both receptors. The affinity of ^{125}I-sCT for the rat C1a receptor is significantly higher than that for the rat C1b receptor,[13] and these receptor isoforms are present in rat brain in similar amounts.[2] Therefore, it is reasonable to assume that the ^{125}I-sCT sites that are labeled in our rat-brain membrane assay are predominantly those of the C1a receptor isoform. Indeed, the IC_{50} value of 0.4 nM obtained for sCT in this assay (Table 4) corresponds well with the K_d value of 0.9 nM reported for sCT binding to the rat C1a receptor, but the K_d value for sCT binding to the C1b receptor is an order of magnitude higher (10 nM).[12] Since hCT and rat calcitonin differ by only two residue substitutions, and the primary structure of the $hCTR_{I1-}$ receptor isoform corresponds most closely to the rat C1a isoform, a close correspondence between the effects of structural changes to hCT on the affinities of these two receptors is perhaps not surprising. Prior studies also point to a correspondence in structure−activity relationships for sCT analogues in binding assays at these two receptors, although the human receptor is reported to be less discriminatory,[44] as indeed it is in our assays.

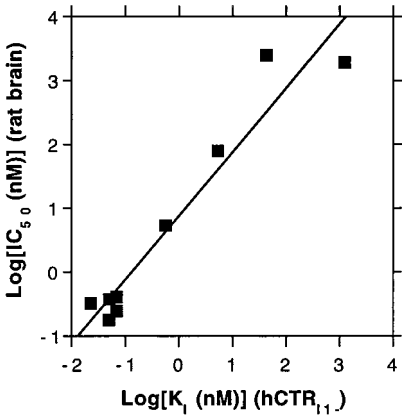

Figure 5. Comparison of peptide potencies in the rat-brain and human calcitonin radioreceptor binding assays. The log values of the IC_{50} concentrations (nM) determined for rat-brain binding are plotted against the log values of K_i (nM) determined for binding to $hCTR_{I1-}$, for peptides S-4, S-2, sCT, S-1, S-3, H-7, H-8, hCT, and H-3 (left-to-right, according to K_i). The solid line represents a linear fit to all of the data.

The activities of sCT and all four of the sCT analogues in this study are very similar (Tables 2−4). This shows quite clearly that the residue substitutions and lactam-bridge conformational constraints introduced into the sCT structure in these analogues have little effect on the affinity of sCT for these receptors or on its agonist potency. In contrast, lactam bridge constraints on residues 17−21 have been demonstrated to enhance the calcitonin-receptor binding affinities and agonist potencies of hCT considerably. In all of the assays used in this study, higher activities were demonstrated for the (Asp^{17}, Orn^{21})-bridged hCT analogue, H-7, in comparison to its unconstrained isosteric analogue H-8 and indeed to hCT itself. Higher in vivo hypocalcemic potencies were also reported earlier for similar lactam-bridged analogues,[16] and we now show that the most potent analogue from those studies, H-2, binds to rat-brain membrane receptors with a higher affinity than hCT (Table 4). These results indicate that increased receptor binding affinity has determined, in large part, the enhanced hypocalcemic potencies reported earlier. They also demonstrate that the receptor binding affinity and agonist potency of sCT and sCT-based analogues have determinants that are different from those of hCT and hCT-based analogues. Prior studies[31] have provided strong evidence that binding of the central amphiphilic α-helical segment of sCT to the extracellular, N-terminal receptor domain is the major determinant of the affinity of sCT for the human calcitonin receptor. This binding interaction may be less important for hCT and hCT-based analogues, since the underlying amino acid sequence shows a lesser propensity for helix formation.

Instead, the affinity of hCT for calcitonin receptors may be determined to a greater extent by additional interactions involving the peptide segments that are constrained within these lactam bridges and/or those that are C-terminal to the bridge constraints and are correctly oriented by the bridges for optimal receptor interactions.

We propose that hCT is predominantly bound to the human and rat calcitonin receptors used in these studies in a conformation that is folded about a tight turn centered around residues 18 and 19. As we have suggested previously,[15] this folded conformation is likely to include a type-I β-turn centered around residues 18 and 19, which has been observed in NMR studies of hCT in DMSO−water solutions.[33] Model-building studies indicate that this turn may be stabilized by appropriate side-chain lactam bridges linking residues 17 and 21, which can mimic the potential H-bonding stabilization of type I β-turns by the native asparagine residue in position 17 of the hCT sequence. Furthermore, the correspondence between the $hCTR_{I1-}$ binding affinities of our hCT analogues, and their agonist potencies, indicates that signal transduction at the human receptor directly involves a state of the calcitonin ligand−receptor complex that requires the lactam-stabilized, folded conformation. In sCT, the folded conformation that we propose for hCT in the G-protein coupled ligand−receptor complex may not form correctly, if sequence differences in the C-terminal sequence in sCT compared to hCT result in a poorer fit with the rat or human receptors. This might offset the advantages of the conformational constraints of the lactam bridges introduced into sCT in analogues S-2, S-3, and S-4. In this case, tighter receptor binding by the sCT-based peptides compared to hCT or any of the hCT analogues that we have studied would then remain dependent on the stronger receptor binding interactions of the amphiphilic helical structure in sCT residues 8−17.

Analogues H-8 and H-7, in that order, exhibit stepwise increases in their receptor binding affinities and agonist potencies compared to hCT (Tables 2−4). This result demonstrates that it is possible to combine potency-enhancing amino acid residue substitutions in hCT with the lactam-bridge constraint of analogue H-2 and produce large, additive effects on receptor affinities and agonist potency. We can speculate that the substitution of Phe^{22} in hCT for Tyr in these analogues is partly responsible for the increased potencies of these analogues, since this substitution is known to increase the hypocalcemic potency of hCT in vivo.[47] In addition, the substitution of Tyr^{12} by Phe represents a decrease in the polarity of the hydrophobic face of the proposed amphiphilic α-helical region, which may enhance the affinity of this structural element for the N-terminal domain of the receptor. Similar hydrophobic substitutions of the leucine residues in this region of the sCT sequence into hCT in corresponding positions are also known to enhance the potencies of hCT,[48,49] presumably by reproducing the tight-binding receptor interactions of sCT in this region. This suggests that there may be additional affinity-enhancing substitutions that may be made to our analogue H-7, in the segment including residues 8−16, that may increase the affinity of this peptide for calcitonin receptors up to and, possibly, beyond that of sCT itself.

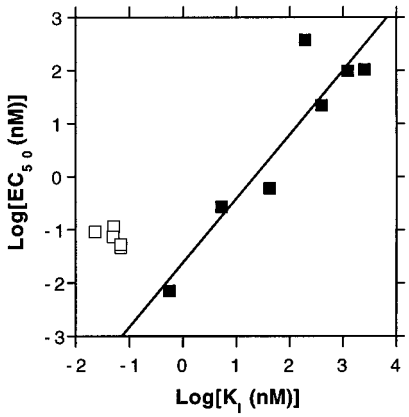

Figure 6. Comparison of peptide activities in the $hCTR_{I1-}$ binding and CRE-luciferase assays. The log values of the EC_{50} (nM) for peptides in the CRE-luciferase assay for signal transduction via $hCTR_{I1-}$ are plotted against the log values of K_i (nM) determined for peptides binding to $hCTR_{I1-}$. Data for peptides sCT, S-1, S-2, S-3, and S-4 are indicated as unfilled squares, and data for peptides hCT, H-1, H-3, H-4, H-5, H-7, and H-8 are indicated as filled squares. The solid line represents a linear fit to the data for hCT and its analogues only.

The data in Tables 2 and 3 indicate that the agonist potency of hCT relative to sCT at $hCTR_{I1-}$ is significantly greater than would be expected on the basis of the relative affinities of these ligands for this receptor: hCT is a full agonist at lower apparent receptor occupancies than is sCT, activating calcitonin receptors more effectively than sCT when it is bound and producing more efficient G-protein release and coupling to the second messenger system. The uniquely wide range of potencies that we have observed for our hCT analogues, including the very high potencies of analogues H-7 and H-8 in particular, allows us to compare this aspect of the agonist character of hCT-sequence-based analogues with that of sCT and its analogues very effectively. A log−log plot of the available EC_{50} and K_i values of all of the analogues that were compared for their affinities and agonist activities at the human insert-negative receptor isoform (Tables 2 and 3) is shown in Figure 6. The linear correlation of these data for the hCT analogues clearly indicates that the stronger agonist character of hCT compared to sCT is retained by the more potent analogues, H-7 and H-8. Conversely, the lower agonist character of sCT, compared to the hCT analogues, is also retained in each of the sCT analogues studied here, as indicated by their positions on this graph off the extrapolated linear relationship shown by the hCT analogues. The most potent hCT analogue, H-7, incorporates only one structural feature taken from the sCT sequence (Tyr^{22}) and still has a lower affinity for $hCTR_{I1-}$ than does sCT. Therefore, we expect that it will be possible to increase the affinity of H-7 for the human receptor still further, by introducing additional affinity-enhancing determinants from the sCT sequence. Incorporation of the Asp-Orn lactam bridge and other single-residue substitutions into hCT to obtain H-7 clearly does compromise the agonist character of hCT. If additional affinity-enhancing determinants from sCT, or others that remain to be identified, can also be introduced into H-7 without altering this essential characteristic of the

hCT-based analogues, then extremely potent agonists at the calcitonin receptor, more than 2 orders of magnitude more potent than sCT, should be readily identifiable. For example, screening analogues of our potent new hCT derivative, H-7, that are substituted in residues 8−16 to increase the affinity of this peptide segment for the N-terminal receptor domain,[31] or substituted in residues 22−32 to decrease dissociation rates for the ligand−receptor complex,[2,50] may be successful approaches toward this goal and may produce novel super-potent hCT analogues with potentially useful therapeutic activities.

Experimental Section

General Methods. Protected L-amino acids were purchased from Bachem and Advanced ChemTech. PyBOP and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl-phenoxyacetamido-norleucyl-MBHA resin (Rink amide MBHA resin) (0.65 mmol/g) were purchased from Novabiochem. HOBt and DCC were purchased from Advanced ChemTech. DIEA (Aldrich, 99.5%) was distilled over ninhydrin. DMF, ^{i}PrOH, and DCM were of synthesis grade from Fisher. ACN from Fisher was of HPLC grade. TFA, EDT, and NMP were from Aldrich. Iodo-beads were from Pierce. Chloramine-T, bacitracin (MW 1422.71, 66,000 units/g), and bovine serum albumin (prepared from Fraction V albumin) were purchased from Sigma. Synthetic sCT and hCT for binding studies were purchased from Advanced ChemTech. 3-Isobutyl-1-methylxantine (IBMX) is from Sigma (Milan, Italy). Tissue culture plastics were purchased from Nunc (Milan, Italy). Media and sera for HEK293-Luc cell culture were purchased from Gibco. Other cell culture medium reagents, including MEM medium, fetal bovine serum (FBS), and nonessential amino acids (NEAA), and the FuGENE transfection reagent were purchased from Life Technologies (San Giuliano Milanese, Italy). Culture plates were from Packard Bioscience (Milan, Italy). For the assays in cultured cells, calcitonin peptides (salmon, human, eel) and amylin were purchased from Novabiochem (Inalco Milan, Italy), and salmon calcitonin 8−32 was from Peninsula Labs, Inc (San Carlos, USA). [^{125}I]-Salmon calcitonin (2000 Ci/mmol) was from Amersham Pharmacia Biotech Inc., Piscataway, NJ, or Amersham Italia Srl, Milan.

Reverse-phase HPLC was performed on a Rainin HPLC system. Peptides were eluted on C_{18} Dynamax-300 Å columns with a 10 μm particle size (4.6 mm × 25 cm for analytical separations, and 10 mm × 25 cm for semipreparative separations). Flow rates were 1.0 mL/min and 3.0 mL/min for the analytical and semipreparative separations, respectively. Eluting solvents used for reverse-phase HPLC were as follows: buffer A, 0.1% (v/v) TFA in deionized water, and buffer B, 0.1% (v/v) TFA in ACN. Peptides were detected at 220 and 276 nm with a Rainin Model UV-D detector. Concentrations of peptide stock solutions in 1 mM HCl were determined by measuring UV absorbance at 274.5 nm using a Beckman DU-65 spectrophotometer. An extinction coefficient of 1440 M^{-1} cm^{-1} was used for all peptides studied.

Peptide Synthesis. Peptides H-1[15] and H-2[16] were synthesized as previously described, and the synthesis of peptide S-4 followed the methods used for H-2. All other peptides were assembled on Rink amide MBHA resin using standard methods for solid-phase peptide syntheses by the Fmoc method,[51] applied and adapted to accommodate the lactam-bridge cyclizations as described herein. L-Amino acids were coupled successively as their N$^{\alpha}$-Fmoc derivatives throughout the syntheses. Reactive side chains of amino acids were protected as follows: Asn(Trt), Asp(OBu^{t}) except Asp^{17}(OPip), Cys(Trt), Gln(Trt), His(Trt), Lys(Boc) except Lys^{21}(Mtt), Ser(Bu^{t}), Orn^{21}-(Mtt), Thr(Bu^{t}), Tyr(Bu^{t}). The coupling reactions, except for those of Pro^{32}, Cys^{1}, and Cys^{7}, were performed with 3 equiv of the protected amino acid, PyBOP, HOBt, and 4.5 equiv of DIEA for about 2 h and their completeness was tested by ninhydrin assay.[51] Cysteine derivatives were coupled using 3 equiv of the preformed symmetrical anhydride in DCM. To attach the C-terminal Pro residue, deprotected Rink amide MBHA resin (0.54 mmol/g) was treated with 0.35 mmol/g resin each of Fmoc-Pro-OH, PyBOP, and HOBT and 0.70 mmol/g resin of DIEA, in DMF (10.0 mL/g resin) for 14 h. The resin was subsequently acetylated with acetic anhydride (3 mmol/g resin) in the presence of DIEA (1.5 mmol/g resin) for 2 h. Substitution levels of 0.25−0.35 mmol/g resin were determined by picric acid analysis.

For some peptides, side-chain lactam-bridge formation, linking the carboxyl side chain of Asp substituted at position 17 with the amine of Orn or Lys in position 21, was performed as follows. The side-chain protecting groups of Asp^{17} and either Lys^{21} or Orn^{21} were simultaneously removed by treatment with a solution of 1% (v/v) TFA and 5% (v/v) TIS in DCM for 1 h. The peptidyl resin was then washed with DCM (×3), ^{i}PrOH (×1), and NMP/DMSO (3/1 [v/v], ×2). Lactam-bridge formation was carried out in NMP/DMSO (3/1 [v/v]) by reaction with 1 equiv of PyBOP in the presence of DIEA at an apparent pH of 8 on wet litmus paper. The reaction mixture was shaken for 5 h, and a second cyclization was performed with fresh reagents for another 5 h. The pH of the reaction mixture was frequently checked during the reaction, and small amounts of DIEA were added to maintain the pH at around pH 8. Completeness of the reaction was tested by ninhydrin assay, which indicated nearly complete reaction in most cases.

After complete peptide chain assembly and N$^{\alpha}$-Fmoc deprotection, peptides were cleaved from the resin in reduced form by treating with TFA/H_2O/EDT/TIS (188/5/5/2 [v/v]) in a round-bottomed flask for 4 h at room temperature. Crude peptide was then precipitated with ice-cold ether, then extracted into 5% (v/v) acetic acid, and lyophilized.

Peptide disulfide bond formation for most analogues was performed by air oxidation as described previously,[16] followed by lyophilization and HPLC purification. However, analogues H-7 and H-8 gave low yields by this method, and were instead oxidized using DMSO as described by Tam and co-workers.[52] Crude reduced peptide (10 mg) was dissolved in 1% (v/v) HOAc (2.0 mL), followed by dilution with distilled water to a volume of 160 mL in a 250 mL beaker. The pH of the peptide solution was adjusted to 6.8 with ammonium carbonate. DMSO (40 mL) was added, and then the reaction mixture was gently stirred for 8 h. Following lyophilization, which usually took 2−3 days, purified, oxidized peptide (approximately 1 mg) was obtained by reversed-phase HPLC. Purity of all peptides was confirmed by analytical HPLC. The correct oxidized structure of each newly synthesized peptide was verified from the average isotopic mass measured by MALDI-TOF MS or EI MS at the University of Michigan Protein and Carbohydrate Structure Facility or from the monoisotopic mass measured by MALDI-TOF using either a PE Biosystems Voyager-DE PRO in reflector mode or a Kratos Kompact MALDI 2, as follows: H-1, average $[M + H]^+ = 3428.6$ (calc. = 3429.0, $\Delta = -0.4$); H-2, average $[M + H]^+ = 3413.7$ (calc. = 3415.0, $\Delta = -1.3$); H-3, monoisotopic $[M + H]^+ = 3385.0$ (calc. = 3385.6, $\Delta = +0.6$); H-4, average $[M + 3H]^{3+} = 1123.8$ (M = 3368.4, calc. = 3367.9, $\Delta = +0.5$); H-5, average $[M + 3H]^{3+} = 1123.3$ (M = 3366.9, calc. = 3367.9, $\Delta = -1.0$); H-6, average $[M + 3H]^{3+} = 1099.9$ (M = 3296.7, calc. = 3296.7, $\Delta = 0.0$); H-7, monoisotopic $[M + H]^+ = 3394.61$ (calc. = 3394.67, $\Delta = -0.1$); H-8, monoisotopic $[M + H]^+ = 3397.05$ (calc. = 3396.67, $\Delta = +0.4$); S-1, average $[M + 5H]^{5+} = 687.2$ (M = 3431.0, calc. = 3430.0, $\Delta = +1.0$); S-2, average $[M + 4H]^{4+} = 858.0$ (M = 3428.0, calc. = 3428.0, $\Delta = 0.0$); S-3, average $[M + H]^+ = 3412.4$ (M = 3411.4, calc. = 3413.9, $\Delta = -2.5$); S-4, average $[M + H]^+ = 3405.9$ (calc. = 3405.9, $\Delta = 0.0$).

Monoiodination of H-7. (a) The chloramine-T method: The method for monoiodination of H-7 was based on that described previously by Taylor et al.[53] Peptide H-7 (100 μL of a 690 μM solution in 1 mM HCl; 69 nmol) was added to 400 μL of phosphate buffer (100 mM NaH_2PO_4/NaOH, pH 6.5) containing 140 nmol NaI. With rapid vortex mixing, aliquots of 50 μL, 25 μL, 12.5 μL, and 12.5 μL of freshly prepared chloramine-T solution (50 μM) were then added at room

temperature at 1 min intervals. One minute after the last addition of chloramine-T, 100 μL of a 1% [w/v] aqueous solution of BSA was added to quench the reaction. The reaction mixture was then directly subjected to HPLC purification using an analytical C_{18} column. The eluting gradient for HPLC purification of iodinated peptide was 32−48% B over 60 min. The HPLC retention time for monoiodinated H-7 was 39.1 min. MALDI-TOF MS gave a monoisotopic MH^+ for monoiodinated H-7 = 3522.39 (calc. = 3521.67, Δ = +0.72).

(b) The iodo-bead method: One iodo-bead was washed twice with phosphate buffer (100 mM NaH_2PO_4/NaOH, pH 6.5) and blotted dry with filter paper before use. The iodo-bead was then incubated in 200 μL of the phosphate buffer containing 14 nmol NaI in a 0.6 mL siliconized microcentrifuge tube at room temperature for 5 min. Peptide H-7 (10 μL of a 690 μM solution in 1 mM HCl; 6.9 nmol) was added. The mixture was shaken for 10 min, and then the iodo-bead was removed from the solution and washed with 100 μL of phosphate buffer. The washings were combined with the reaction mixture and subjected to HPLC purification, as before.

$HCTR_{I1-}$ Expression and Assay of Radioreceptor Binding. HEK293-Luc cells, which derive from human embryonic kidney HEK293 cells by stable expression of a cAMP-responsive MRE-CRE luciferase gene-reporter construct,[42] were stably transfected with an expression plasmid (pcDNA 3.1/Zeo/GC2) containing the $hCTR_{I1-}$ cDNA and a gene conferring resistance to Zeocin. The cells were cultured following adhesion to polylysine-coated tissue-culture plates and maintained in an EMEM medium containing L-glutamine (2 mM) supplemented with 10% (v/v) fetal-calf serum, 0.5 mg/mL active Geneticin (G-418), and 0.4 mg/mL zeocin.

Membranes were prepared by lysis in hypotonic phosphate buffer, using a modification of the method described by Scheideler and Zukin.[54] Cells were harvested in phosphate-buffered saline (approximately 3×10^7 cells/30 mL tube) and collected by centrifugation at 800*g* for 5 min. Each pellet was then resuspended in 10 mM potassium phosphate buffer, pH 7.2 (buffer A; 30 mL/pellet), and centrifuged at 40000*g* for 10 min. The pellets obtained were resuspended in the same volume of buffer A, incubated on ice for 20 min, and centrifuged at 1200 rpm for 5 min, saving the supernatants. The low-speed pellets were resuspended in buffer A again, and the last step was repeated two more times, saving the supernatants each time. The low-speed supernatants were pooled and centrifuged at high speed, as before. The pellets obtained were then resuspended in buffer A containing 0.32 M sucrose and 5 mM EDTA (buffer B). The membranes were washed and concentrated in this storage buffer at 2−5 mg protein/mL (ca. 1×10^7 cells/mL), and the aliquots were frozen at −80 °C.

Binding experiments using the membranes from HEK293-Luc cells stably expressing $hCTR_{I1-}$ were performed in 25 mM potassium phosphate buffer, pH 7.4, containing 3 mM $MgCl_2$, 0.1% (w/v) BSA, bacitracin (0.5 mg/mL), leupeptin (4 μg/mL), and chimostatin (2 μg/mL). Nonspecific binding was determined in the presence of 1 μM unlabeled sCT. Incubation was carried out for 60 min at 25 °C in a final volume of 0.5 mL. The reaction was terminated by filtration using a Packard Filtermate Harvester containing a GF/B Unifilter plate pretreated with 0.3% (w/v) polyethylenimine. After filtration, the Unifilter plates were washed two times with 1 mL of cold incubation buffer and dried. Then each well was filled with 50 μL of Packard Microscint 30 and counted using a Packard Topcount NXT.

Competitive radioligand displacement experiments were performed using a membrane concentration of 40−60 μg protein/mL and radioligand concentrations close to the experimental K_d obtained from saturation binding isotherms. IC_{50} values were determined using the nonlinear least-squares fitting program, GraFit (Erithacus Software Limited, Horley, U.K.),[55] and were then transformed into K_i values by applying the equation of Cheng and Prusoff.[56] Analysis of the saturation binding experiments was performed using LIGAND.[40]

CRE-Luciferase Gene-Reporter Assay. Human neuroblastoma SH-SY5Y cells were received from Dr. T. Flannigan (SB Oxford University, Psychoneuropathology unit, U.K.) and maintained in MEM medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 1% (w/v) NEEA at 37 °C in a humidified atmosphere with 5% CO_2. Receptor activation by the $hCTR_{I1-}$ ligands was assessed in SH-SY5Y after cotransfection with equal amounts (14 μg/4 $\times$ 10^6 cells in 10 cm dishes) of $hCTR_{I1-}$ cDNA in pCDNA 3.1/zeo and a cAMP-responsive MRE/CRE luciferase gene-reporter construct.[42] Transfection was performed using FuGENE 6 transfection reagent (Roche, Indianapolis, IN), as recommended by the manufacturer. Twenty-four hours after transfection, cells were recovered from the dishes by gentle trypsinization and plated (5×10^4 cells/well) in 96-well Culture plates (Packard) in 70 μL of culture medium. After 24 h incubation at 37 °C, cells were used in receptor signaling assays, employing the CRE-luciferase gene-reporter assay, as previously described by Fitzgerald et al.[42] Briefly, cells were preincubated for 30 min in the presence of 0.5 mM phosphodiesterase inhibitor IBMX. Drugs were then added as 10-fold concentrated solutions as appropriate, in a final volume of 100 μL. Luciferase activity was measured after incubation for 4 h. An equal volume of reconstituted LucLite reagent was added to each well, then solutions were incubated in the dark for 10 min at room temperature and counted in a TopCount Microplate Scintillation and Luminescence counter (Packard Bioscience, Milan, Italy). The amount of luciferase expressed in each well, which is proportional to the luminescence (CPS) measured, was expressed as fold-increase over basal levels. Data were analyzed by nonlinear fitting, using GraphPad Prism (v 2.01; GraphPad, San Diego, CA) to determine EC_{50} values.

Rat-Brain Membrane Radioreceptor Binding Assays. Rat-brain membrane preparations and binding assays were based on the methods described by Nakamuta et al.[57] and Kapurniotu et al.[15] Brain membranes were prepared from male Sprague−Dawley rats (250−350 g) and stored in ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing freshly added 0.32 M sucrose and 34 μM (48 μg/mL or 3.2 units/mL) bacitracin (6 mL buffer per whole brain). The resulting rat-brain tissue homogenate was aliquoted into 1.0 mL portions for storage at −65 °C. Protein concentrations were measured using the micro-Lowry protein assay kit from Sigma (St. Louis, MO) based on the method of Peterson,[58] with BSA as a standard. The final protein content in each microcentrifuge assay tube was approximately 0.75 mg/mL.

Binding assays were carried out in ice, in an assay buffer consisting of 50 mM Tris-HCl, pH 7.4, containing freshly added bacitracin (34 μM). All tubes (1.5 mL siliconized microcentrifuge tubes) and pipet tips used for binding assays were soaked in assay buffer for 4 h at room temperature and blotted dry with a paper towel without washing. For two binding assays, three tubes of frozen brain preparations were thawed and diluted to 50 mL with ice-cold assay buffer. Stock solutions of competing peptides in 1 mM HCl were diluted to the appropriate concentrations immediately before the binding assay. In each tube, 20 μL [^{125}I-Tyr^{22}]sCT (10 pM, final concentration) was added, followed by addition of 20 μL of competing peptides, and then 960 μL of the chilled rat-brain membrane suspension (approximately 0.63 mg of protein per tube). Incubation was started by the addition of the chilled brain membrane preparation and continued by shaking on ice for 30 min. The binding was terminated by centrifugation of the tubes at room temperature for 10 min. After discarding the supernatants, the membrane pellets were washed with 200 μL of ice-cold assay buffer and recentrifuged for 4 min. The tips of the assay tubes containing the pallets were cut off and placed into scintillation vials containing 0.5 mL of 2% (w/v) SDS in water, and then the scintillation vials were shaken at room temperature for at least 4 h to solubilize the pellets. Vials were then counted in added scintillation fluid (3 mL ScintiSafe Econo 1), using a Beckman LS 6500 liquid scintillation counter. Nonspecific [^{125}I-Tyr^{22}]sCT binding was determined in the presence of 1 μM sCT. Specific binding was determined as the difference between total binding and nonspecific binding, and IC_{50} values were determined by probit analysis.

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References

(1) Azria, M. *The Calcitonins: Physiology and Pharmacology*; KARGER: Basel, 1989; pp 133−143.

(2) Sexton, P. M.; Findlay, D. M.; Martin, T. J. Calcitonin. *Curr. Med. Chem.* **1999**, *6*, 1067−1093.

(3) Braga, P. C. Calcitonin and its antinociceptive activity: animal and human investigations 1975−1992. *Agents Actions* **1994**, *41*, 121−131.

(4) Hilton, J. M.; Mitchelhill, K. I.; Pozvek, G.; Dowton, M.; Quiza, M.; Sexton, P. M. Purification of calcitonin-like peptides from rat brain and pituitary. *Endocrinology* **1998**, *139*, 982−992.

(5) Kaiser, E. T.; Kézdy, F. J. Amphiphilic Secondary Structure: Design of Peptide Hormones. *Science* **1984**, *223*, 249−255.

(6) Abbreviations: BSA, bovine serum albumin; hCTR, human calcitonin receptor; DCM, dichloromethane; DMF, dimethylformamide; DIEA, *N*,*N*′-diisopropylethylamine; EDTA, *N*,*N*,*N*′,*N*′-ethylenediamine(tetraacetic acid); EI, electrospray ionization; FMOC, 9-fluorenylmethoxycarbonyl; GPCR, G-protein coupled receptor; hCT, human calcitonin; IBMX, 3-isobutyl-1-methylxantine; MALDI, matrix-assisted laser-desorption ionization; MBHA, *p*-methylbenzhydrylamine; OFm, fluoren-9-ylmethylester; RIA, radioimmunoassay; sCT, salmon calcitonin; TCA, tricloroacetic acid; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TOF, time-of flight.

(7) Findlay, D. M.; Martin, T. J. Receptors of calciotropic hormones. *Horm. Metab. Res.* **1997**, *29*, 128−134.

(8) Houssami, S.; Findlay, D. M.; Brady, C. L.; Myers, D. E.; Martin, T. J.; Sexton, P. M. Isoforms of the rat calcitonin receptor: consequences for ligand binding and signal transduction. *Endocrinology* **1994**, *135*, 183−190.

(9) Bergwitz, C.; Gardella, T. J.; Flannery, M. R.; Potts, J. T., Jr.; Kronenberg, H. M.; Goldring, S. R.; Jüppner, H. Full activation of chimeric receptors by hybrids between parathyroid hormone and calcitonin. *J. Biol. Chem.* **1996**, *271*, 26469−26472.

(10) Kuestner, R. E.; Elrod, R. D.; Grant, F. J.; Hagen, F. S.; Kuijper, J. L.; Matthewes, S. L.; O'Hara, P. J.; Sheppard, P. O.; Stroop, S. D.; Thompson, D. L.; Whitmore, D. E.; Findlay, D. M.; Houssami, S.; Sexton, P. M.; Moore, E. E. Cloning and characterization of an abundant subtype of the human calcitonin receptor. *Mol. Pharmacol.* **1994**, *46*, 246−255.

(11) Gorn, A. H.; Lin, H. Y.; Yamin, M.; Auron, P. E.; Flannery, M. R.; Tapp, D. R.; Manning, C. A.; Lodish, H. F.; Krane, S. M.; Goldring, S. R. Cloning, characterization, and expression of a human calcitonin receptor from an ovarian carcinoma cell line. *J. Clin. Invest.* **1992**, *90*, 1726−1735.

(12) Sexton, P. M.; Houssami, S.; Hilton, J. M.; O'Keefe, L. M.; Center, R. J.; Gillespie, M. T.; Darcy, P.; Findlay, D. M. Identification of brain isoforms of the rat calcitonin receptor. *Mol. Endocrinol.* **1993**, *7*, 815−821.

(13) Albrandt, K.; Mull, E.; Brady, E. M.; Herich, J.; Moore, C. X.; Beaumont, K. Molecular cloning of two receptors from rat brain with high affinity for salmon calcitonin. *FEBS Lett.* **1993**, *325*, 225−232.

(14) Moore, E. E.; Kuestner, R. E.; Stroop, S. D.; Grant, F. J.; Matthewes, S. L.; Brady, C. L.; Sexton, P. M.; Findlay, D. M. Functionally different isoforms of the human calcitonin receptor result from alternative splicing of the gene transcript. *Mol. Endocrinol.* **1995**, *9*, 959−968.

(15) Kapurniotu, A.; Taylor, J. W. Structural and Conformational Requirements for Human Calcitonin Activity: Design, Synthesis, and Study of Lactam-Bridged Analogues. *J. Med. Chem.* **1995**, *38*, 836−847.

(16) Kapurniotu, A.; Kayed, R.; Taylor, J. W.; Voelter, W. Rational design, conformational studies and bioactivity of highly potent conformationally constrained calcitonin analogues. *Eur. J. Biochem.* **1999**, *265*, 606−618.

(17) Felix, A. M.; Heimer, E. P.; Wang, C. T.; Lambros, T. J.; Fournier, A.; Mowles, T. F.; Maines, S.; Campbell, R. M.; Wegrzynski, B. B.; Toome, V.; Fry, D.; Madison, V. S. Synthesis, biological activity and conformational analysis of cyclic GRF analogs. *Int. J. Pept. Protein Res.* **1988**, *32*, 441−454.

(18) Krstenansky, J. L.; Zechel, C.; Trivedi, D.; Hruby, V. J. Importance of the C-terminal alpha-helical structure for glucagon's biological activity. *Int. J. Pept. Protein Res.* **1988**, *32*, 468−475.

(19) Condon, S. M.; Morize, I.; Darnbrough, S.; Burns, C. J.; Miller, B. E.; Uhl, J.; Burke, K.; Jariwala, N.; Locke, K.; Krolikowski, P. H.; Kumar, N. V.; Labaudiniere, R. F. The bioactive conformation of human parathyroid hormone. Structural evidence for the extended helix postulate. *J. Am. Chem. Soc.* **2000**, *122*, 3007−3014.

(20) Greenberg, Z.; Bisello, A.; Mierke, D. F.; Rosenblatt, M.; and Chorev, M. Mapping the bimolecular interface of the parathyroid hormone (PTH)-PTH1 receptor complex: spatial proximity between Lys(27) (of the hormone principal binding domain) and leu(261) (of the first extracellular loop) of the human PTH1 receptor. *Biochemistry* **2000**, *39*, 8142−8152.

(21) Schievano, E.; Mammi, S.; Bisello, A.; Rosenblatt, M.; Chorev, M.; Peggion E. Conformational studies of a bicyclic, lactam-constrained parathyroid hormone-related protein-derived agonist. *J. Pept. Sci.* **1999**, *5*, 330−337.

(22) Taylor, J. W.; Kaiser, E. T. The structural characterization of β-endorphin and related peptide hormones and neuropeptides. *Pharmacol. Rev.* **1986**, *38*, 291−319.

(23) Meadows, R. P.; Nikonowicz, E. P.; Jones, C. R.; Bastian, J. W.; Gorenstein, D. G. Two-dimensional NMR and structure determination of salmon calcitonin in methanol. *Biochemistry* **1991**, *30*, 1247−1254.

(24) Meyer, J. P.; Pelton, J. T.; Hoflack, J.; Saudek, V. Solution structure of salmon calcitonin. *Biopolymers* **1991**, *31*, 233−241.

(25) Katahira, R.; Doi, M.; Kyogoku, Y.; Yamada-Nosaka, A.; Yamasaki, K.; Takai, M.; Kobayashi, Y. Solution structure of a human calcitonin analog elucidated by NMR and distance geometry calculations. *Int. J. Pept. Protein Res.* **1995**, *45*, 305−311.

(26) Epand, R. M.; Epand, R. F.; Orlowski, R. C. Presence of an amphiphatic helical segment and its relationship to biological potency of calcitonin analogs. *Int. J. Pept. Protein Res.* **1985**, *25*, 105−111.

(27) Siligardi, G.; Samori, B.; Melandri, S.; Visconti, M.; Drake, A. F. Correlations between biological activities and conformational properties for human, salmon, eel, porcine calcitonins and elcatonin elucidated by CD spectroscopy. *Eur. J. Biochem.* **1994**, *221*, 1117−1125.

(28) Epand, R. M.; Epand R. F. Conformational Flexibility and Biological Activity of Salmon Calcitonin. *Biochemistry* **1986**, *25*, 1964−1968.

(29) Epand, R. M.; Epand, R. F.; Orlowski, R. C. Biologically active calcitonin analogs which have minimal interactions with phospholipids. *Biochem. Biophys. Res. Commun.* **1988**, *152*, 203−207.

(30) Nakamuta, H.; Orlowski, R. C.; Epand, R. M. Evidence for calcitonin receptor heterogeneity: binding studies with non-helical analogues. *Endocrinology* **1990**, *127*, 163−169.

(31) Stroop, S. D.; Nakamuta, H.; Kuestner, R. E.; Moore, E. E.; Epand; R. M. Determinants for calcitonin analog interaction with the calcitonin receptor N-terminus and transmembrane-loop regions. *Endocrinology* **1996**, *137*, 4752−4756.

(32) Ösapay, G.; Taylor, J. W. Multicyclic Polypeptide Model Compounds. 2. Synthesis and Conformational Properties of a Highly α-Helical Uncosapeptide Constrained by Three Side-Chain to Side-Chain Lactam Bridges. *J. Am. Chem. Soc.* **1992**, *114*, 6966−6973.

(33) Motta, A.; Temussi, P. A.; Wünsch, E.; Bovermann, G. A ^{1}H NMR Study of Human Calcitonin in Solution. *Biochemistry* **1991**, *30*, 2364−2371.

(34) Richardson, J. S. The anatomy and taxonomy of protein structure. *Adv. Protein Chem.* **1981**, *34*, 167−339.

(35) Stanger, H. E.; Gellman, S. H. Rules for antiparallel β-sheet design: D-Pro-Gly is superior to L-Asn-Gly for β-hairpin nucleation. *J. Am. Chem. Soc.* **1998**, *120*, 4236−4237.

(36) Cohen, D. P.; Nussenzveig, D. R.; Gershenghorn, M. C. Iodocalcitonin binds to human calcitonin receptors with higher affinity than calcitonin. *Endocrinology* **1996**, *137*, 4507−4510.

(37) Greenfield, N. J. Methods to estimate the conformation of proteins and polypeptides from circular dichroism data. *Anal. Biochem.* **1996**, *235*, 1−10.

(38) Chen, Y.-H.; Yang, J. T.; Chau, K. H. Determination of the helix and beta form of proteins in aqueous solution by circular dichroism. *Biochemistry* **1974**, *13*, 3350−3359.

(39) Arvinte, T.; Cudd, A.; Drake, A. F. The structure and mechanism of formation of human calcitonin fibrils. *J. Biol. Chem.* **1993**, *268*, 6415−6422.

(40) Munson, P. J.; Rodbard, D. Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **1980**, *107*, 220−239.

(41) Christopoulos, G., Perry, K. J., Morfis, M., Tilakaratne, N., Gao, Y., Fraser, N. J., Main, M. J., Foord, S. M., and Sexton, P. M. Multiple amylin receptors arise from receptor activity-modifyling protein interaction with the calcitonin receptor gene product. *Mol. Pharmacol.* **1999**, *56*, 235−242.

(42) Fitzgerald, L. R.; Mannan, I. J.; Dytko, G. M.; Wu, H.-L.; Nambi, P. Measurement of responses from Gi-, Gs-, or Gq-coupled receptors by a multiple response element/cAMP response element-directed reporter assay. *Anal. Biochem.* **1999**, *275*, 54−61.

(43) Gorn A. H.; Rudolph S. M.; Flannery M. R.; Morton C. C.; Weremowicz S.; Wand J. T.; Krane S. M.; Goldring S. R. Expression of two human skeletal calcitonin receptor isoforms cloned from a giant cell tumor of bone. *J. Clin. Invest.* **1995**, *95*, 2680−2691.
(44) Pozvek, G.; Hilton, J. M.; Quiza, M.; Houssami, S.; Sexton, P. M. Structure/function relationships of calcitonin analogues as agonists, antagonists, or inverse agonists in a constitutively activated receptor cell system. *Mol. Pharmacol.* **1997**, *51*, 658−665.
(45) Fischer, J. A.; Sagar, S. M.; Martin, J. B. Characterization and regional distribution of calcitonin binding sites in the rat brain. *Life Sci.* **1981**, *29*, 663−671.
(46) Rizzo, A. J.; Goltzman, D. Calcitonin Receptors in the Central Nervous System of the Rat. *Endocrinology* **1981**, *108*, 1672−1677.
(47) Maier, R.; Kamber, B.; Riniker, B.; Rittal, W. Analogues of human calcitonin II. Influence of modifications in amino acid positions 1, 8 and 22 on hypocalcemic activity in the rat. *Horm. Metab. Res.* **1975**, *7*, 511−514.
(48) Maier, R.; Kamber, B.; Riniker, B.; Rittel, W. Analogues of human calcitonin. IV. Influence of leucine substitutions in positions 12, 16 and 19 on hypocalcaemic activity in the rat. *Clin. Endocrinol.* **1976**, *5s*, 327s−332s.
(49) Basava, C.; Hostetler, K. Y. Structural determinants for the design of superpotent analogs of human calcitonin. In *Peptides: Chemistry and Biology 1991*; Smith, J. A., Rivier, J. E., Eds.; ESCOM Science Publishers B. V.: Leiden, 1992; pp 20−22.
(50) Hilton, J. M.; Dowton, M.; Houssami, S.; Sexton, P. M. Identification of key components in the irreversibility of salmon calcitonin binding to calcitonin receptors. *J. Endocrinol.* **2000**, *166*, 213−226.
(51) Stewart, J. M.; Young, J. D. *Solid-Phase peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1984; pp 53−123.
(52) Tam, J. P.; Wu, C.; Liu, W.; Zhang, J. Disulfide Bridge Formation in Peptides by Dimethyl Sulfoxide. *J. Am. Chem. Soc.* **1991**, *113*, 6657−6662.
(53) Taylor, J. W.; Bidard, J.; Lazdunski, M. The Characterization of High-Affinity Binding Sites in Rat Brain for the Mast Cell-degranulating Peptide from Bee Venom Using the Purified Monoiodinated Peptide. *J. Biol. Chem.* **1984**, *259*, 13957−13967.
(54) Scheideler, M. A.; Zukin, R. S. Reconstitution of solubilized delta-opiate receptor binding sites in lipid vesicles. *J. Biol. Chem.* **1990**, *265*, 15176−15182.
(55) Leatherbarrow, R. J. Using linear and nonlinear regression to fit biochemical data. *Trends Biochem. Sci.* **1990**, *15*, 455−458.
(56) Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099−3108.
(57) Nakamuta, H.; Furukawa, S.; Koida, M.; Yajima, H.; Orlowski, R.; Schlueter, R. Specific Binding of 125I-Salmon Calcitonin to Rat Brain: Regional Variation and Calcitonin Specificity. *Jpn. J. Pharmacol.* **1981**, *31*, 53−60.
(58) Peterson, G. L. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **1977**, *83*, 346−356.

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