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N-TERMINAL TRUNCATION OF SALMON CALCITONIN LEADS TO CALCITONIN ANTAGONISTS

Structure Activity Relationship of N-terminally Truncated Salmon Calcitonin Fragments In Vitro and In Vivo

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SUMMARY: Structural requirements for binding to the bone calcitonin (CT) receptor and for CT bioactivity both in vitro and in vivo were assessed for a series of N-terminally truncated, N^{α}-acetylated, fragments of salmon calcitonin (sCT). Sequential deletion of amino acid residues from the amino-terminus of [Ala^7]sCT-(2-32) peptide amide first led to partial agonists and, upon deletion of residues 1 to 7, to a high affinity antagonist, N^{α}-acetyl-sCT-(8-32)-NH_2. The presence of two separate domains within the sCT sequence is proposed: (I) a binding domain comprising residues 9-32 and (II) an activation domain requiring residues 3 to 6. N^{α}-acetyl-sCT-(8-32)-NH_2, in several bioassays including plasminogen activator release from LLC-PK1 cells ($pA_2 = 7.31$), cAMP production in UMR-106-06 cells ($pA_2 = 7.81$), and in the fetal rat long bone resorption assay showed potent antagonistic properties.

The main biological effect of calcitonin (CT), a 32 amino acid polypeptide hormone, is to inhibit osteoclastic bone resorption, acting directly on osteoclasts to inhibit their activity [1] and to prevent fusion of osteoclast-precursors via a specific CT receptor [2-3]. All the known calcitonins consist of a 32 amino acid single peptide chain ending in proline amide and containing a cyclic disulfide bridge between residues 1 and 7 [4]. Structure activity relations have suggested that the biological activity is dependent on the integrity of the cyclic structure [5-7]. In the meantime, linear analogues of salmon CT have been reported to retain full biological activity, suggesting that the ring structure is not required in salmon CT [8-9].

MATERIALS AND METHODS

Peptide Synthesis: Peptides were synthesized by a segment-coupling approach. N-acetyl-sCT-(n-9) segments were assembled using Fmoc chemistry on a p-hydroxymethylphenyloxy-methyl-polystyrene support and coupling by N,N'-diisopropyl-carbodiimide and hydroxy-benzotriazol in DMF. Segments were cleaved from the support by TFA and coupled to the

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partially protected segment, H-[11,18-Lys(Boc),15-Glu(OtBu)]-sCT-(10-32) amide [10] using DCCI and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBT). Remaining protecting groups were removed with 90% TFA and peptides purified by C-18-RP HPLC. Final products showed correct amino acid ratios after hydrolysis in 6 N HCL and the expected molecular ions in FAB-MS.

Radio Receptor Assay: Binding studies were performed using a crude bone cell suspension prepared from neonatal rat long bones as described [11]. Binding buffer consisted of 10 mM HEPES, 130 mM choline-chloride, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, 10 µg/ml of bacitracin, 2 µM PMSF and 100 TIU/ml of aprotinin, pH 7.5. 200 µl of the bone cell suspension (approx. 10^7 cells/ml) were incubated (2 h, 20 °C) with 30,000 cpm of [^{125}I]sCT (mono-iodinated 3-[^{125}I]sCT, spec. act. of 81 TBq/mmol) in the presence or absence of the competing ligand (final volume 300 µl). The incubation was terminated by adding 1 ml of washing buffer (10 mM TRIS, 150 mM NaCl, 4 °C), and cells were harvested on Whatman GF/B glass-fibre filters.

cAMP Production: cAMP production was measured in confluent cultures of the rat osteosarcoma cell line UMR 106-06 using a radiometric assay [12]. Intracellular [^{3}H]cAMP was determined using sequential Dowex 50W-X4 and alumina chromatography [13].

Plasminogen Activator (PA) Release: LLC-PK1 kidney cells were grown to confluence in 96 well plates [14]. PA activity was measured by a colorimetric assay of the plasmin-dependent hydrolysis of D-Val-Leu-Lys-4-nitroanilide under serum free conditions [15]. Absorbance was determined at 405 nm.

Bone Resorption: Nineteen-day-old fetal rat forelimb bones, previously labelled in utero with ^{45}Ca, were dissected and cultured as described previously [16]. Bone resorbing activity was expressed as the percentage of total bone ^{45}Ca released at day 2 and expressed as the treatment/control ratio.

Hypocalcaemic Activity In Vivo: The hypocalcaemic activity of the calcitonin analogues (0.2 µg/kg, i.v.) was determined by continuous measurements of Ca ionized blood levels in 5-weeks-old rabbits (body weight approx. 0.7 kg) using the selective ion electrode [17].

Data Analysis: Competition data were analyzed using SCTFIT, a non-linear regression computer program for the analysis receptor binding data [18]. Antagonistic activity (pA_2 value) was calculated by the method of Arunlakshana and Schild [19].

RESULTS / DISCUSSION

In the present investigation the structure activity relations in a series of linear, N-terminally truncated N^{α}-acetyl-[Ala^7]-sCT peptide amide fragments were established (these fragments will be referred to as sCT-(X-32) fragments, X = 2 to 9). Sequential amino acid deletions yielding peptides as short as the sCT-(9-32) fragment did not affect the receptor binding affinity. The only exception in this series was the sCT-(5-32) fragment showing a binding affinity of 40 times less compared to sCT (see Table 1). These results confirm and extend previous findings that linear sCT analogues do recognize the calcitonin receptor [8,9] and that the presence of the N-terminal ring structure formed by the disulfide bridge between position 1 and 7 is not a prerequisite in retaining high affinity. Complete removal of the ring moiety

Table 1: Structure activity relation of N^{α}-acetyl-[Ala^7]sCT peptide amide fragments

sCT Fragments	*Receptor Affinity*	*Plasminogen Activator Release LLC-PK-1 cells*		*Cyclic AMP Production UMR 106-06 cells*		*Hypocalcemia Rabbit*
	IC_{50} (nM)	EC_{50} (nM)	i.a.*	EC_{50} (nM)	i.a.*	Rel. Potency**
salmon Calcitonin (sCT)	0.2	1.18 ± 0.37	1.0	0.93 ± 0.48	1.0	100
N^{α}-acetyl-[Ala^7]sCT-(2-32) amide	0.9	1.87 ± 0.56	0.94	0.46 ± 0.01	1.0	105
N^{α}-acetyl-[Ala^7]sCT-(3-32) amide	1.2	2.18 ± 0.91	0.96	1.49 ± 0.60	1.0	67
N^{α}-acetyl-[Ala^7]sCT-(4-32) amide	1.0	2.64 ± 1.78	0.93	2.20 ± 1.40	0.98	72
N^{α}-acetyl-[Ala^7]sCT-(5-32) amide	13.5	25.9 ± 8.3	0.61	8.10 ± 1.12	0.11	52
N^{α}-acetyl-[Ala^7]sCT-(7-32) amide	0.9	71.1 ± 23.7	0.07	>10000	<0.01	0
N^{α}-acetyl-sCT-(8-32) amide	0.5	>10000	<0.01	>10000	<0.01	n.d.
N^{α}-acetyl-sCT-(9-32) amide	0.8	>10000	<0.01	>10000	<0.01	n.d.
N^{α}-acetyl-sCT-(10-32) amide	160.0	>10000	<0.01	>10000	<0.01	0

* intrinsic acivity (i.a.): sCT = 1 by definition,
** acute hypocalcemic activity in rabbit in vivo: sCT = 100 by definition.

gave sCT-(9-32) fragment, which showed a minor loss (4-fold) in affinity compared to sCT. Further removal of 9-leucine gave sCT-(10-32) fragment, which showed a dramatic 800-fold decrease in affinity. This observation indicated that the leucine residue at position 9 must be retained in order to preserve high affinity binding to the CT receptor. From the binding data it is concluded that the 9-32 domain of sCT contains all the determinants for high affinity interaction with the CT receptor.

Biological activity of the fragments was assessed in vitro by activation of cAMP production in rat osteosarcoma cells and stimulation of plasminogen activator (PA) release from a pig kidney LLC-PK1 cell line (Table 1). In contradistinction to high affinity binding, bioactivity declined substantially when 4 or more amino acids were removed from the N-terminus of sCT. Both in the PA release assay (sCT; EC_{50} = 1.2 ± 0.37 nM) and the cAMP assay in UMR-106-06 (sCT; EC_{50} = 0.9 ± 0.48 nM) full biological activity was retained up to the sCT-(4-32) fragment. Further removal of amino acid residues at position 4 or from 4 to 6 resulted in a loss of potency in the PA assay, EC_{50} values of 25.9 nM and 71.1 nM respectively. Partial agonism was apparent by the gradual loss of intrinsic activity (i.a.) in these com-pounds. Partial agonism was also demonstrated in the cAMP assay for the sCT-(5-32) fragment (EC_{50} 8,1 nM, i.a. 0.11), while the sCT-(7-32) fragment was inactive. The sCT-(8-32) fragment, identified as a high affinity compound in the binding assay, was completely devoid of biological activity in both in vitro assay systems.

Testing for hypocalcaemic activity in the rabbit, we found a similar structure activity relationship as observed in the in vitro bioassays. Removal of the first two amino acid residues preserved full bioactivity (i.v., 0.2 µg/kg body weight; sCT dose with maximal

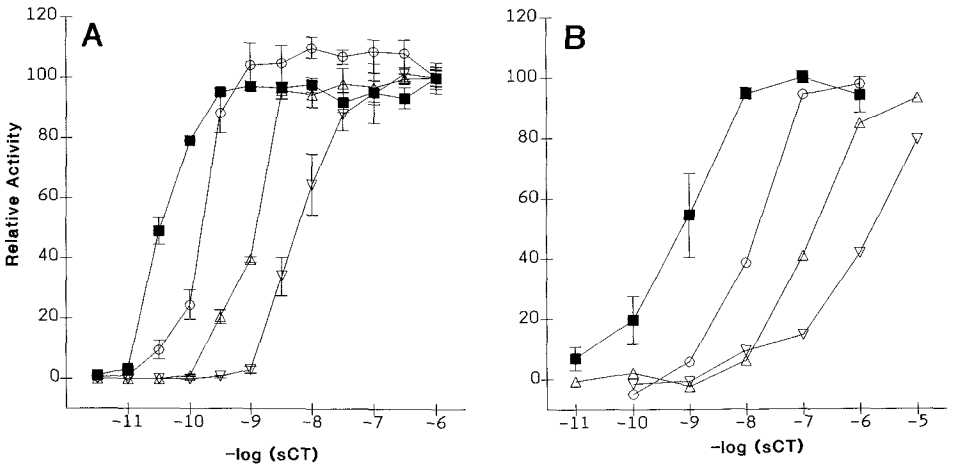

Figure 1. Effect of N^{α}-acetyl-sCT-(8-32) peptide amide fragment (sCT-(8-32) fragment) on the sCT-stimulated release of plasminogen activator from LLC-PK-1 cells (panel A) and on the stimulation of cAMP production in UMR-106-06 cells (panel B) as described in Materials and Methods. Cells were incubated with increasing concentrations of sCT alone (black squares) or in combination with fixed, progressively increasing concentrations of sCT-(8-32) fragment (10^{-7} M, circle; 10^{-6} M, triangle; 10^{-5} M, inverted triangle).
Results are presented as % stimulation of the maximal response.

hypocalcaemic effect). However, further removal of the 3-asparagine resulted in a 33% drop in bioactivity compared to sCT, and similar results were found for (5-32) and (6-32) fragments. The sCT-(7-32) fragment showed no hypocalcaemic activity in the rabbit at this dose (see Table 1).

To further explore a possible antagonistic activity of the sCT-(8-32) fragment, concentration/dose-response studies of sCT were performed in the presence of this analogue. Both in the cAMP assay and the PA-release assay, increasing concentrations of the antagonist caused a parallel shift to the right in the sCT dose-response curves without decreasing the maximal effect as shown in figure 1. Schild-plot analysis of the shifts of the curves in the cAMP assay and the PA release assays gave pA_2 values of 7.81 (slope of 1.07) and 7.31

Table 2: Effect of N^{α}-acetyl-sCT-(8-32) peptide amide fragment (sCT-(8-32) fragment) on the sCT-induced inhibition of the release of ^{45}Ca in fetal rat long explants

	sCT without sCT-(8-32)	*sCT with 10^{-6} M of sCT-(8-32)*
IC_{50} value	0.015 nM	32 nM

Fetal rat long bones were prepared and cultured as described in Materials and Methods. The PTH-stimulated (10^{-8} M) release of ^{45}Ca into the culture medium was inhibited by sCT, in the presence or absence of 10^{-6} M of sCT-(8-32) fragment. The bone resorption rate was expressed as the percentage release of total bone ^{45}Ca released after two days of culture. Dose-response curves were analyzed using the mean value ± SEM of two separated experiments (n = 6), and presented as EC_{50} values.

(slope of 0.9) respectively. In the rat long bone resorption assay, sCT inhibited the PTH-stimulated (10^{-8} M) release of ^{45}Ca into the culture medium. This effect was antagonized in the presence of 10^{-6} M of sCT-(8-32) fragment (see Table 2). Despite the observed antagonistic properties in the functional assays in vitro, so far no antagonism could be demonstrated in vivo; sCT-(8-32) fragment (1 mg/kg body weight) had no effect on the calcium lowering activity of sCT in thyroparathyroidectomized (TPTX) rats (results not shown). Preliminary findings using a more stable analogue of sCT-(8-32) fragment clearly showed antagonistic features in vivo, suggesting that the lack of an effect of the sCT-(8-32) fragment is possibly due to a short half-life in the organism (to be published).

In accordance with the finding that the integrity of the ring structure at the N-terminus of sCT is not required to maintain a high affinity interaction between the ligand and the receptor, linear and/or N-terminally truncated sCT analogues showed full biological responses both in vitro and in vivo. Furthermore, our results suggest that two separate domains are located within the amino acid sequence of sCT. All the determinants for binding of sCT to the receptor are located in the 9-32 sequence. From the biological results it is concluded that the minimal sequence for activation of the receptor (activation domain) resides in amino acid residues 3 to 6. It should be noted that the cysteine and serine residues at positions 1 and 2 and, therefore, the integrity of the N-terminal ring structure is not essential for full bioactivity. Sequential deletion beyond amino acid residue in position 3 leads to partial agonists and eventually to a pure antagonist.

Structural similarities between the calcitonin receptor, the parathyroid hormone receptor and the secretin receptor have led to the proposal of a new class of seven-transmembrane-spanning G protein coupled receptors [3]. In this context it is striking to observe that sequential deletions at the amino-termini of the corresponding hormones, in all cases, lead to a change in profile from full agonists to partial agonists to pure antagonists [20,21, this study]. Thus, one may postulate that the observed regularity in the structure activity relationship of these hormones reflects a distinctive feature of the structural organization of this class of peptide receptors.

In conclusion, the structure activity data of a series of N-terminally truncated N^{α}-acetyl-[Ala^7]-sCT peptide amide fragments led us to suggest two separate domains within the sCT sequence: (I), a binding domain located in the 9-32 sequence and (II), an activation domain with the minimal required sequence allocated to amino acid residues in positions 3 to 6.

N^{α}-acetyl-sCT-(8-32) peptide amide (code name SDZ 212-524) was identified as a high affinity sCT analogue with potent antagonistic properties in several bioassays in vitro. Further characterization of more stable CT antagonists will allow us to better define the function(s) of calcitonin in vivo.

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