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ORIGINAL PAPER

# A synthetic peptide derived from mouse pituitary calcitonin cDNA sequence exhibits potent inhibition of prolactin secretion and prolactin mRNA abundance in primary mouse pituitary cells

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**Abstract** We have shown that gonadotrophs synthesize and secrete immunoreactive calcitonin (CT)-like peptide, and CT is a potent inhibitor of prolactin (PRL) secretion and gene transcription. CT cDNA cloned from LßT2 cells (pit-CT cDNA) exhibits 99% homology with mouse CT cDNA sequence, but exhibits four mismatches in the coding region of CT peptide (347–485 bp) with consequent changes in the amino acids at positions 5 and 17 of mouse CT. We have synthesized a putative 23 amino acid pit-CT peptide based on pit-CT cDNA sequence, and tested its effect on PRL secretion and mRNA abundance in primary mouse pituitary cells. The results suggest that synthetic pit-CT attenuates PRL mRNA abundance and inhibits PRL release from mouse anterior pituitary cells. Moreover, pit-CT is remarkably more potent than salmon (S)CT in attenuating PRL mRNA abundance. These results raise a possibility that this endogenous pituitary peptide may potentially serve as a therapeutic molecule for the treatment of prolactinomas.

**Keywords** Prolactin · mRNA suppression · Synthetic pit-CT

## Introduction

Calcitonins (CTs) are a group of polypeptide hormones containing 32 amino acids secreted by C cells of the thyroid gland [1–5]. Besides the thyroid gland, CTs show a wide distribution in the CNS, pituitary gland [6–9], and several other organs like the thymus, lungs, jejunum, kidney, urinary bladder [10, 11], uterine endometrium [12, 13], mammary gland [14], and the prostate [15, 16] in all mammalian species studied so far. Receptors for CT have also been found in these organs [17–20]. Previous studies from this laboratory have shown that CT-like immunoreactive peptide is synthesized and released by gonadotrophs of rat anterior pituitary (AP) gland [15]. Subsequently, the pituitary CT (pit-CT) cDNA was cloned from a mouse gonadotroph-derived LßT2 cell line. The cloned CT cDNA referred to as pit-CT in this manuscript exhibited 99% homology with mouse thyroid CT cDNA sequence [21]. However, the sequence exhibits four mismatches in the coding region of the mature pit-CT peptide (347–485 bp). These mismatches would result in the changes in the amino acid residues of pit-CT at positions 5 and 17 when compared with mouse thyroid CT peptide. In pit-CT peptide (derived from pit-CT cDNA sequence) cysteine residues at positions 1 and 7 from N-terminus, a unique feature of the CT family of peptides, are retained. However, the proline residue at position 32 of thyroid CT has been eliminated as a result of the mismatch of a nucleotide at that position in the pit-CT cDNA sequence, leading to the truncation of C-terminal region of the putative pit-CT peptide [21].

A series of studies from this and other laboratories have demonstrated that exogenously added synthetic sCT potently inhibits PRL biosynthesis and release [15, 22–27], PRL mRNA abundance and gene transcription [28], lactotrope cell proliferation [29] and PRL promoter activity [30]. Moreover, rabbit anti-CT serum immunoneutralizes endogenous pit-CT, stimulates PRL release from cultured AP cells, and increases serum PRL levels in ovariectomized adult rats [21, 27]. These results, when combined with our other results that CT like immunoreactive peptide is synthesized and released by gonadotrophs of the

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pituitary gland [15, 21], raise a strong possibility that gonadotroph-derived CT is an important paracrine regulator of lactotrope-PRL system.

The objective of the present studies was to test whether the synthetic peptide derived from L$\beta$T2-cloned pit-CT cDNA sequence (pit-CT) mimics the actions of SCT on PRL cells by inhibiting PRL release and PRL mRNA abundance in mouse AP cells. Fig. 1

## Results

### Effect of pit-CT on PRL release

The results of Fig. 2 suggest that inhibitory effect of CT peptides on PRL release was rapid in onset, but stabilized in 30 min, at which point both CT peptides (10 nM) were equipotent (Fig. 2A, B and C). The next experiment examined dose-response relationship of CT peptides on PRL release after 30 min of incubation. The results suggest that both CT peptides caused a significant inhibition of PRL release at concentrations of 10 pM or greater (Fig. 3). Although both peptides produced maximal inhibition of approximately 51% over basal levels, pit-CT seemed somewhat more potent at subnanomolar concentrations.

| Position: | 1-10 | 11-20 | 21-32 |
|---|---|---|---|
| Pit-CT: | CGNL**T**TCMLG | TYTQDL**H**KFH | TFP |
| Mouse-CT: | CGNL**S**TCMLG | TYTQDL**N**KFH | TFPQTSIGVEAP-amide |

Serine at position 5 is replaced with Threonine

Asparagine at position 17 is replaced with histidine

Aminoacids 24-32 are removed.

**Fig. 1** Primary amino acid sequences of mouse CT and pit-CT, which were derived from their cDNA sequences as reported in Ren et al. [21]. The differences in the sequences are also highlighted

### Effect of pit-CT on PRL mRNA

We also examined the effect of CT peptides on PRL mRNA levels because our earlier studies have shown that SCT is more efficient in inhibiting PRL gene transcription than PRL release [28]. Results of quantitative RT-PCR depicted in Fig. 4 demonstrate that pit-CT mimicked the action of SCT by attenuating PRL mRNA abundance in mouse AP cells. However, pit-CT was remarkably more potent than SCT. A maximal reduction of 76% was achieved by SCT (100 nM), and a comparative decrease by equimolar pit-CT was 92%. More significantly, the inhibitory effect of pit-CT on PRL mRNA abundance became evident at concentrations as low as 0.1 pM causing around 34% inhibition (EC50 = 0.1 pM). In contrast, the inhibitory effect of SCT could be observed only in nanomolar range (Fig. 4A and B). These results suggest that pit-CT is

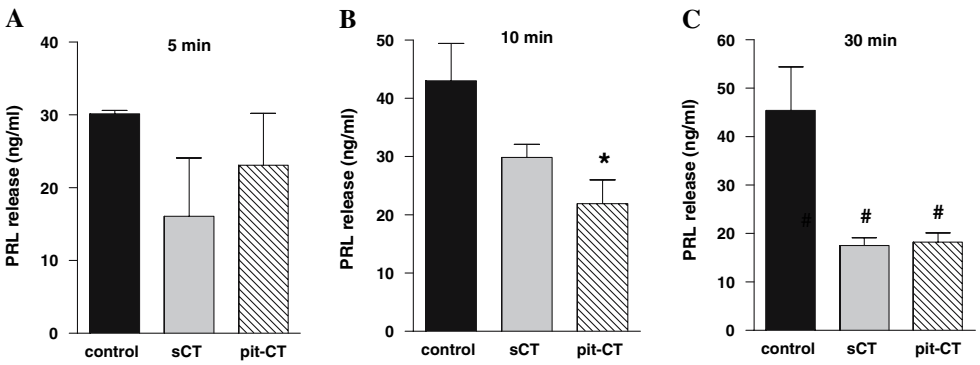


**Fig. 2** Effect of 10 nM concentrations of pit-CT and SCT on PRL release (in ng/ml) from cultured mouse AP cells after (**A**) 5, (**B**) 10 and (**C**) 30 min post-treatment. *$P < 0.05$ vs. control and #$P<0.01$ vs. control, (one-way analysis of variance [ANOVA] and Newman-Keuls multiple comparison test)

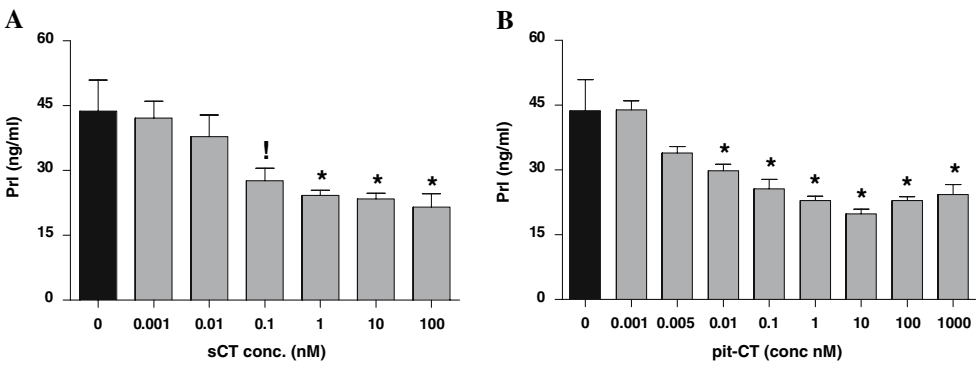


**Fig. 3** Effect of various concentrations of (**A**) SCT *$P < 0.01$ vs. control and !$P < 0.05$ vs. control, (**B**) pit-CT *$P < 0.001$ vs. control and 0.001 nM, (one-way analysis of variance [ANOVA] and Newman-Keuls multiple comparison test) on PRL release (in ng/ml) from cultured mouse AP cells 30 min post-treatment

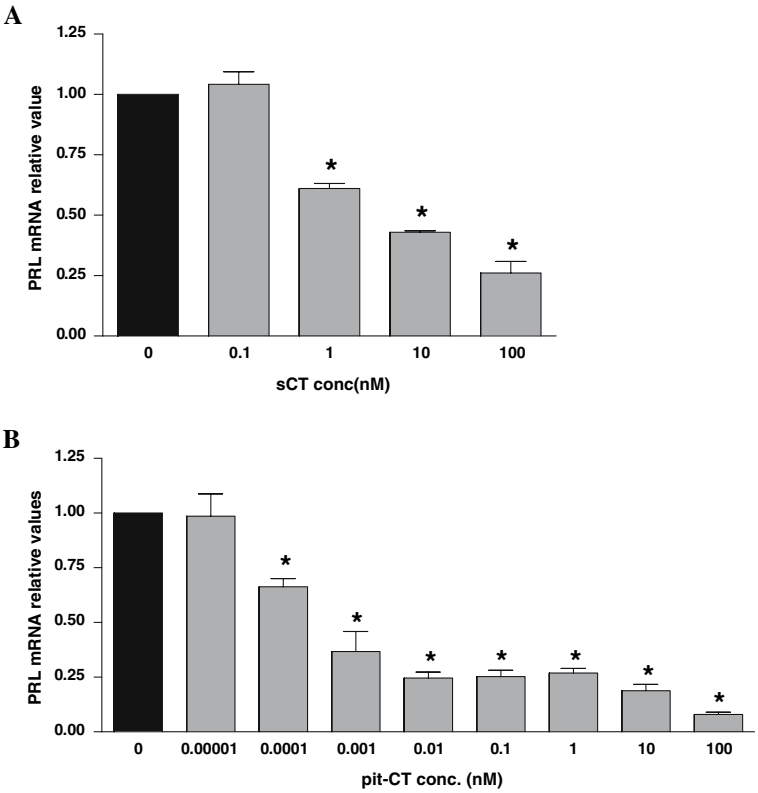


**Fig. 4** Effect of various concentrations of (**A**) SCT and (**B**) pit-CT on PRL mRNA abundance (expressed as a relative value of the mean for control) in cultured mouse AP cells, 12 h following treatment. *$P < 0.001$ vs. control, (one-way analysis of variance [ANOVA] and Newman-Keuls multiple comparison test)

**Table 1** Inhibition of prolactin mRNA levels in mouse anterior pituitary cultures by synthetic pit-CT versus sCT

| Peptide concentration | % inhibition of PRL mRNA form mouse primary AP cells | |
|---|---|---|
| | Pit-CT | sCT |
| 1 pM | 34.0 | – |
| 1 nM | 72.9 | 38.8 |
| 10 nM | 81.2 | 56.9 |

Effect of different concentrations of synthetic pituitary derived calcitonin (pit-CT) and salmon CT (SCT) on PRL mRNA expression after 12 h of treatment time in cultured primary mouse AP cells. Results from RT-PCR expressed as percent inhibition compared to the control.

more potent than SCT in inhibiting PRL gene expression in primary mouse AP cells (Table 1).

## Discussion

Accumulating evidence suggests that the pituitary gland synthesizes and secretes number of neuroendocrine peptides and growth factors such as TGF-α, EGF, TGF-β3, bFGF, IGF, NGF, VIP, galanin, neurotensin, TGF-β1, TNF-α, and CT [31, 32]. Receptors for these peptides have also been identified in a variety of target cells within the anterior pituitary gland [33–36], suggesting the regulation of pituitary function occurring at paracrine, juxtacrine, and autocrine level through the action of these intra-pituitary peptides [15, 21, 27, 37].

Our previous studies have demonstrated that CT is biosynthesized and secreted by gonadotrophs in the anterior pituitary gland [15, 21]. In addition, there are also reports of the presence of SCT-like immunoreactive peptide in the mammalian pituitaries including rodents and humans [15, 38, 39]. Moreover, both SCT and HCT (or rat CT) have been shown to interact with the same CTR, and SCT is generally found to be more potent than HCT in most biological assays [40–43]. Since the mRNA sequence of pit-CT was closely-related to mouse CT mRNA sequence, we decided to compare the biological activity of pit-CT with SCT, a selective and potent inhibitor of PRL biosynthesis, secretion and lactotroph cell proliferation [15, 22–26], which may also serve as an agonist for endogenous SCT-like intra-pituitary peptide [9, 44–46]. The present results have shown that both peptides were almost equipotent in inhibiting PRL release from AP cells. However, pit-CT was remarkably more potent than SCT in attenuating PRL mRNA abundance, suggesting pit-CT may differentially activate CTR or post-receptor signaling pathway(s). The present results are consistent with the phenotype of our pit-CT transgenic mice, where we have selectively targeted the overexpression of CT in gonadotrophs. The heterozygous mice displayed remarkable loss of lactotrophs, a dramatic decrease in PRL mRNA

abundance and chronic hypoprolactinemia. However, homozygous mice were almost aprolactinemic and infertile, suggesting the dosing effect of the transgene expression [37]. Additional studies will be necessary to examine the role of C-terminal region as well as substitutions of amino acid residues at positions 5 and 17 in interactions with the CTR and activation of post-receptor signaling mechanisms.

While PRL is a key hormone for normal reproductive function, excess of PRL is associated with several dysfunctions like infertility, amenorrhea, galactorrhea, anovulation, and luteal phase insufficiency in females, and in males impotence, infertility, and hypogonadism are observed [47]. Prolactinoma, a tumor resulting from hypersecretion of PRL producing cells is a major disease affecting women [48]. The drugs currently utilized in treating prolactinoma like dopamine agonists; bromocriptine and cabergoline although very effective, also cause several serious side effects. Findings from a recent report suggest that about 15–20% of prolactinomas are resistant to dopamine on account of the loss of dopamine receptors or due to faulty signaling pathways [49]. Therefore there is an urgent need to identify and characterize novel inhibitors of PRL secretion like pit-CT that can offer improved treatments for prolactinomas by better efficacy and lesser undesirable side effects.

## Materials and methods

### Materials

Dulbeccos modified eagle medium (DMEM), penicillin G-streptomycin sulfate mixture, trypsin, DNase I, horse and fetal calf sera, and superscript II ™ reverse transcriptase were purchased from Invitrogen (Carlsbad, CA). iQ SYBR Green Supermix was obtained from Bio-Rad Laboratories (Hercules, CA). PCR primers were synthesized by Genemed Synthesis Inc. (South San Francisco, CA). Mouse PRL was purchased from Dr. A. F. Parlow (National Hormone and Peptide Program, Torrance, CA), $^{125}$I-sodium iodide was purchased from PerkinElmer Inc. (Waltham, Massachusetts), other reagents for PRL RIA were a generous gift from Dr. Frank Talamantes (UC Santa Cruz, CA). All other chemicals were obtained from Sigma chemical Co. (St. Louis, MO).

### Animals

Six- to eight-week-old adult female C57BL/6 mice obtained from Jackson Laboratories (Bar Harbor, ME) were housed on a 12/12 h light/dark cycle with ad libitum access to food and water. Their estrous cycle was monitored by vaginal cytology and the anterior pituitary glands were harvested on the morning of proestrus.

### Preparation of primary cultures of anterior pituitary cells

Anterior Pituitary glands were freshly harvested on the morning of proestrus by rapid decapitation and transferred to serum free DMEM supplemented with 0.3% BSA, 10 mM HEPES, 10 μg/ml gentamycin, and 2.8 mg/ml bacitracin and minced into small pieces. Single cells were prepared by enzymic (trypsin-DNase) dispersion, as previously described [15]. The cells were then plated at a density of 40–50,000 cells per well in 96-well culture plates in DMEM with 5% heat inactivated FBS, 15% heat inactivated horse serum, and antibiotics and cultured for 48 h at 37 C in 95% air-5% $CO_2$.

### Radioimmunoassay for PRL in the conditioned media

Fourty-eight hours after plating, the primary AP cells were serum starved for 2 h, and then treated with 10 nM of SCT and pit-CT, separately, for 5′, 10′, and 30′ ($n$ = 3 wells per time point for each treatment group) and incubated at 37 C. At the end of incubation period, the conditioned media were collected and stored at −80°C, until analyzed. The PRL levels in the conditioned media were measured using the mouse PRL RIA reagents kindly provided by Dr. Frank Talamantes (University of California, Santacruz, CA). All samples from the experiment were run in duplicates in the same RIA. The bound hormone was separated from the free hormone by using protein A. The sensitivity of the assay was 0.1953 ng/ml. The results are presented as ng/ml of PRL released by 50,000-cells/well + /− SEM after 30 min. A maximal response was observed within 30′. So in subsequent experiment a dose response study with 30 min treatment time was performed, with both the peptides and control ($n$ = 3 wells per concentration for each treatment group), and the PRL content in the conditioned media was measured in duplicates for all the samples as described above in three separate experiments.

### Extraction of mRNA

The cultured mouse anterior pituitary cells were treated with various concentrations of SCT and pit-CT ($n$ = 6 wells for each concentration of each peptide, and control) for a period of 12 h, and total RNA was extracted as described by Xie and Rothblum [50]. Briefly, total RNA

from AP cells was extracted by pooling extracts from two wells as one sample, thus generating three samples for each concentration of either peptide and control, using a one-step acid-guanidinium thiocynate–phenol–chloroform extraction method. It was then precipitated with isopropanol, washed with 70% ethanol and resuspended in DEPC-treated water.

### Reverse transcription of mRNA and quantitation by real time PCR

One microgram of total RNA was used for reverse transcription (RT). Oligo dT primer annealing and RT were performed using Superscript II reverse transcriptase, and the manufacturers instructions were followed. Real time PCR for GAPDH and PRL was carried out using SYBR Green super mix on an I-cycler with the following sets of primer pairs for PRL and GAPDH respectively:

mouse PRL forward primer 5′-TCTCAGGCCATCTT GGAGAAGTGTG-3′ mouse PRL reverse primer 5′-GGGCAGTCATTGATGACCTTGACCA-3′ and mouse GAPDH forward primer 5′-ACAGCCGCATCTTCTTG TGC-3′ mouse GAPDH reverse primer 5′-GCCTCACC CCATTTGATGTT-3′.

The thermal cycling parameters used for PCR were set as below:

To determine the annealing temperature for each set of primers, the reactions were performed over a gradient of several temperatures (data not shown). The temperature providing most amplicons was selected for all subsequent reactions for that set of primers. One cycle with a hold step at 50 C for 2 min, and an activation step for DNA polymerase at 95°C for 3 min and 30 s, followed by 40 cycles each of 15 s at 95°C for denaturation and 1 min anneal/extension period at 65°C for GAPDH and 66.8°C for PRL, respectively. The levels of fluorescence in each well ($n$ = 3 wells/sample for each of the three samples per treatment group) were monitored continuously throughout the 40 cycles of amplification. PRL mRNA levels were normalized to GAPDH mRNA content and expressed as a relative value of the mean for control. The entire experiment was repeated two more times, and each experiment consisted of triplicates for each group.

## Data analysis

### For RIA

The results from three separate experiments for PRL RIA were statistically evaluated using one-way analysis of variance (ANOVA), and significance was derived using Newman-Keuls multiple comparison test.

### Quantitation of RT PCR

The data from quantitative real time PCR were analyzed using the relative quantification technique that provides a means for comparing the amount of mRNA in each group of sample (treatment) with the amount in the control group using the $2^{-\Delta\Delta CT}$ method. The CT values from each experiment consisted of a mean value of the triplicates ran for each treatment group. Such CT values from three separate experiments were analyzed by one-way analysis of variance [ANOVA] and Newman-Keuls multiple comparison test.

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