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Calcitonin-Suppressed Expression of Parathyroid Hormone-Related Protein in Breast Cancer Cells

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Parathyroid hormone-related protein (PTHrP) is a key factor behind humoral hypercalcemia of malignancy (HHM). It is produced in most breast tumors and may be an important local mediator of skeletal metastases due to breast cancer. PTHrP may mediate local bone destruction in the absence of increased circulating PTHrP. Calcitonin (CT) is used for treatment of HHM, but there are data showing that CT can increase PTHrP expression and secretion *in vitro*. We have therefore studied the effect of CT on PTHrP gene expression and secretion in MCF-7 breast cancer cells. PTHrP mRNA decreased significantly after 4, 8, and 16 h incubation with 10 nM salmon calcitonin (sCT) when compared with the respective controls. PTHrP mRNA also decreased significantly and dose-dependently after incubation with sCT at 0.1 to 10 nM for 16 h. The PTHrP levels in the conditioned medium also decreased in a similar dose-dependent manner. The adenylate cyclase agonist forskolin lowered the PTHrP mRNA dose-dependently. In cells exposed to varying concentrations of sCT for 15 min, the cAMP levels increased dose-dependently. In conclusion, sCT can suppress PTHrP gene expression in MCF-7 breast cancer cells. The suppressive effect is probably exerted mainly via the cAMP–protein kinase A pathways.

Key Words: **breast cancer; calcitonin; cAMP; gene expression; parathyroid hormone-related protein.**

The hypercalcemic effect of parathyroid hormone-related protein (PTHrP) is exerted via the PTH/PTHrP receptors in bone and kidney. The role of PTHrP in the pathogenesis of humoral hypercalcemia of malignancy (HHM) in patients with solid tumors has been well studied [1–3], and high levels of PTHrP have been found in the circulation of most patients with HHM [4, 5]. PTHrP production has been demonstrated both in normal and malignant breast tissue [6, 7] and several studies have shown that increased PTHrP production in breast cancer tumors is related to the incidence of skeletal metastases [8–10].

Calcitonin (CT), a 32-amino-acid peptide hormone which inhibits osteoclastic activity [11], can antagonize the hypercalcemic effects of PTH and PTHrP and is sometimes used in the treatment of HHM [12, 13]. *In vitro* studies using a lung cancer cell line (BEN cells) have demonstrated that CT increases PTHrP gene expression and secretion [14, 15]. However, the effect of CT on PTHrP production in breast cancer cells is unknown.

MCF-7, a PTHrP-secreting breast cancer cell line [16], expresses a specific CT receptor and CT-responsive adenylate cyclase [17]. The aim of the present study was to investigate influence of CT on PTHrP expression and secretion in MCF-7 cells.

MATERIALS AND METHODS

Cell culture. MCF-7 cells were cultivated to confluence in Dulbecco's minimum essential medium (DMEM) (Life Technologies AB, Stockholm, Sweden) supplemented with 10% of fetal bovine serum (FBS), 100 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine in flasks of 162 cm^2. The propagated cells were seeded at a density of 5×10^5 cells/well. After subconfluence, the cells were incubated with DMEM without FBS for 24 h before treatment with salmon calcitonin (sCT) (E. Sandoz, Hanover, NJ) or forskolin (Sigma, St. Louis, MO). For the controls in Fig. 4, the same amount of ethanol, which was the vehicle for forskolin, was added.

RNA extraction and reverse transcription. After collection of the conditioned medium, the cells were lysed intact in the culture wells with 500 μl of a solution containing 4 M guanidinium thiocyanate, 0.75 M sodium citrate, 10% sarcosyl and 0.1 mM 2-mercaptoethanol [18]. The lysates were transferred into Eppendorf tubes and 500 μl of a 125:24:1 mixture of phenol, chloroform and isoamyl alcohol (Sigma) was added. After centrifugation, the RNA in the supernatant was precipitated with isopropanol and washed in cold ethanol (75%). The RNA pellet was dissolved in RNase-free water processed with diethylpyrocarbonate (Sigma) and denatured at 65°C. By use of a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Boehringer-Mannheim, Germany), 0.5 μg of RNA sample was applied into a reverse transcription reaction tube in a total volume of 20 μl with final concentrations of 5 mM $MgCl_2$, 10 mM Tris, 50 mM KCl, 1 mM

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dNTP Mix, 0.04 A_{260} units of Oligo-p$(dT)_{15}$ primer, 50 units of RNase inhibitor and 20 units of AMV reverse transcriptase. After reverse transcription at 42°C for 60 min, the reaction tubes were heated at 99°C for 5 min in order to denature the AMV enzyme, and thereafter it was immediately cooled on ice. The resulted cDNA was applied to PCR amplification.

Quantitative competitive PCR (QC-PCR). Following reverse transcription, PTHrP mRNA was determined by a recently developed quantitative competitive PCR assay [19] and expressed as copies per microgram of total cellular RNA. In brief, PTHrP cDNA in the samples, in parallel to the known amount of PTHrP cDNA standard (0, 10, 10^2, 10^3, 10^4, and 10^5 copies), was amplified in the presence of a competitor DNA sequence (10^3 copies/tube). The PCR volume was 25 μl, containing 0.5 μM upstream (biotinylated) and downstream primers, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.65 U Taq DNA polymerase (PCR Master reagent from Boehringer-Mannheim, Germany). The cycling profile was 1 min at 94°C, 1 min at 60°C and 1 min at 72°C for 35 cycles in a programmable thermal controller PTC-100-96V (M. J. Research, Inc., Watertown, MA). The PCR products were immobilized in streptavidin-coated microtitration wells in duplicate. One aliquot was detected by an Eu-labeled target probe and the other by an Eu-labeled competitor probe. The ratio of target to competitor fluorescence signal counts was used for calculation. Based on the linear regression equation derived from the standard curve, the PTHrP mRNA copies in the RNA samples were determined. To compensate for variations in RNA degradation and reverse transcription, the level of β-actin mRNA was used to correct the determined PTHrP mRNA.

Internal control for PTHrP QC-PCR. A competitive RT-PCR assay for determination of the relative amount of β-actin mRNA in the RNA samples has been developed in a similar way as the QC-PCR assay for PTHrP mRNA [19]. A pair of primers spanning 540 base-pairs (bp) of β-actin cDNA [20] was designed. The primers were located on different exons of the human β-actin gene [21] to prevent the problems of genomic DNA carry-over contamination. The upstream primer was biotinylated at the 5′-end. By use of a pair of specially designed primers (Table 1), a sequence of 80 bp in the middle of the target β-actin cDNA fragment was deleted and substituted with a sequence of 21 bp, which resulted in a competitor DNA of 481 bp for β-actin cDNA PCR. The inserted sequence of 21 bp is the same as that in the PTHrP competitor DNA, and therefore they can be detected with the same Eu-labeled probe following separate PCRs. An Eu-labeled 21-mer oligonucleotide probe (Table 1) hybridizing to the midregion of amplified β-actin cDNA fragment was used for detection of PCR product of the target β-actin cDNA.

In the presence of 2×10^6 β-actin competitor DNA molecules per PCR tube, 2.5 μl of each RT reaction was applied into a PCR of 25 μl containing: 0.2 μM of upstream and downstream primers (Table 1), and 12.5 μl of PCR Master (Boehringer-Mannheim, Germany). The cycling profile was 1 min at 94°C, 1 min at 52°C and 1 min at 72°C for 30 cycles. The PCR products were then immobilized in streptavidin-coated wells. The β-actin and competitive cDNAs were detected by separate Eu-labeled probes and ratio of target to competitor fluorescence signal counts was used to correct the PTHrP mRNA values in each sample respectively.

PTHrP immunofluorometric assay. The conditioned medium in the presence of protease inhibitors (10 μl/ml of a solution containing 10000 KIE/ml of Trasylol, and 0.5 M EDTA) was frozen at −70°C until analysis. PTHrP in the conditioned medium was measured by a time-resolved lanthanide immunofluorometric assay (IFMA) with a detection limit of 0.3 pM. The intra-assay coefficients of variation were 9.6% at 0.3, 7.5% at 0.6 and 4.5% at 1.3 pM ($n = 6$). The interassay coefficients of variation were 14.3% at 2.5, 6.4% at 10, and 11.4% at 20 pM ($n = 6$) [22]. In brief, 0.2 ml of the medium was incubated overnight at 4°C with biotinylated anti-PTHrP 38-67 rabbit antibody immobilized in streptavidin-coated wells of a microtitration plate (Wallac Oy, Turku, Finland). After washing, 0.2 ml of Eu-labeled anti-PTHrP 1-34 sheep antibody was added and the plate was shaken for 2 h at room temperature. After stringent washing, the fluorescence signal, generated after addition of 0.15 ml of enhancement solution per well, was measured by a DELFIA 1234 plate fluorometer.

TABLE 1

Primers for Constructing the Competitor DNA of β-Actin cDNA and for Performing PCR and Probes Labeled with Eu-Chelate for Detecting PCR Products (5′-3′)

Construction of competitor DNA
For 1st fragment
P-1, GTGGGGCGCCCCCAGGCACCA (113–132)
P-2, GTTATCCGCTCACAATTTCGGTCAGCAGCACG (330–315)
For 2nd fragment
P-3, GTGAGCGGATAACAATTTGGCCATCCAGGCTG (411–425)
P-4, CTCCTTAATGTCACGCACGATTTC (652–629)
PCR amplification
Upstream primer
Biotin-GTGGGGCGCCCCCAGGCACCA (113–132)
Downstream primer
CTCCTTAATGTCACGCACGATTTC (652–629)
Probes for Eu-chelate labeling
Target DNA probe
NH-GGTCATCTTCTCGCGGTTGGC (370–350)
Competitor DNA probe
NH-AATTGTTATCCGCTCACAATT (inserted sequence)

Note. The underlined sequences are the single-stranded terminals that are joined during a splice overlap extension PCR.

Cyclic AMP extraction and radioimmunoassay. The cells were incubated with 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX, Sigma) in DMEM without FBS for 1 h and then treated with sCT, or forskolin for 15 min in DMEM together with 0.5 mM of IBMX. After removal of the medium, intracellular cAMP was extracted by ice-cold 90% propanol at 4°C overnight. The propanol extract was centrifuged for 5 min at 3000 rpm, and after desiccation of the supernatant, the extracts were frozen at −20°C for later cAMP analysis with a BIOTRAK cAMP radioimmunoassay system (Amersham Life Science, Buckinghamshire, UK). The detection limit is 25 fmol/tube and the values were standardized to fmol/μg cellular protein.

Total cellular protein assay. The cells were dissolved in 500 μl of 1 M NaOH and heated at 100°C for 1 min. After neutralization with 500 μl of 1 M acetic acid, 100 μl of the solution was added to 700 μl of phosphate buffered saline together with 200 μl of a protein assay reagent (Bio-Rad, CA). After 5 min the absorbance at 595 nm was determined in a spectrophotometer. Known amounts of transferrin were treated in the same way and used as a standard.

Statistics. The data are presented as mean ± SEM (standard error of the mean) in the figures and the statistical significance was analyzed by one-way ANOVA compared with the respective control group (Figs. 1, 2 and 4) or *t* test (Fig. 3). *P* values <0.05 were regarded to be significant.

RESULTS

After 16 h incubation with 0.1–10 nM sCT, PTHrP mRNA was significantly decreased from 125,000 copies (control) to 40,000 copies (10 nM) per microgram of total RNA in a dose-dependent manner (Fig. 1A). After incubation of the cells with 1 to 100 μM of forskolin, the PTHrP mRNA was also decreased dose-dependently (Fig. 2). The PTHrP levels in the conditioned medium decreased in a similar way (Fig. 1B).

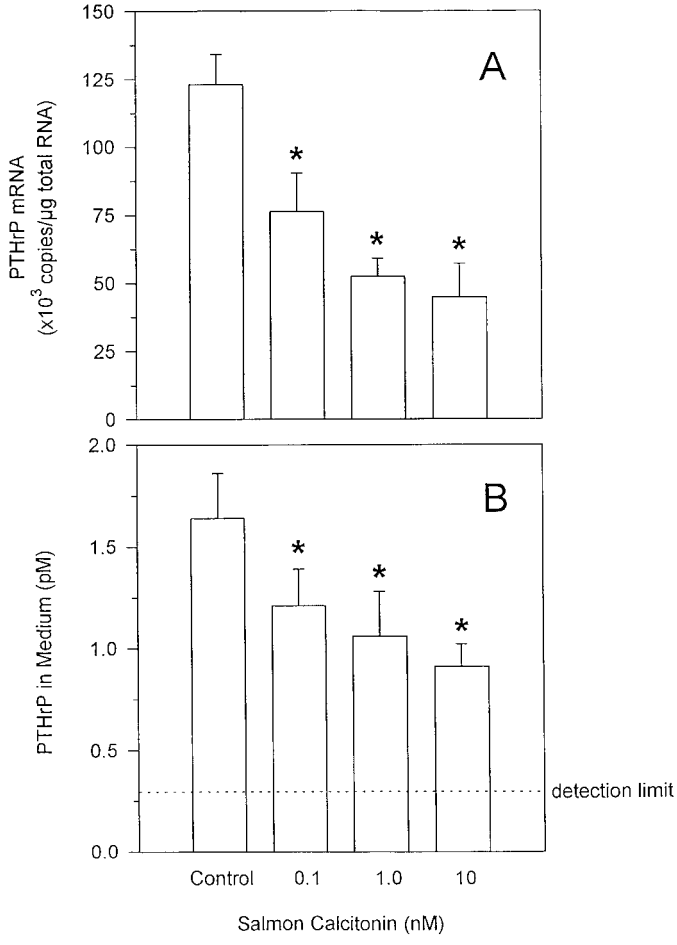

FIG. 1. PTHrP mRNA in MCF-7 breast cancer cells (A) and PTHrP levels in the conditioned medium (B) after incubation with 0.1–10 nM salmon calcitonin for 16 h ($n = 6$). *$P < 0.05$ when compared with the control group.

After treatment of the cells with 10 nM of sCT for 2, 4, 8 and 16 h, the PTHrP mRNA expression varied at different time points in both control and treatment groups but the PTHrP mRNA concentration was significantly lower in the cells treated with sCT at all time points compared to the respective control groups (Fig. 3). The highest expression was found after 4 h incubation and the expression was decreased when the incubation period was prolonged.

The intracellular cAMP levels were significantly and dose-dependently increased after stimulation with 0.1 to 10 nM salmon calcitonin (Fig. 4). Forskolin (40 μM), which was used as a positive control, increased cAMP levels from 31 $\pm$ 3 (control) to 2028 $\pm$ 174 fmol/μg protein. All figures show one out of two experiments with similar results.

DISCUSSION

The PTHrP gene expression in normal mammary adenoepithelial cells and in breast adenocarcinorma

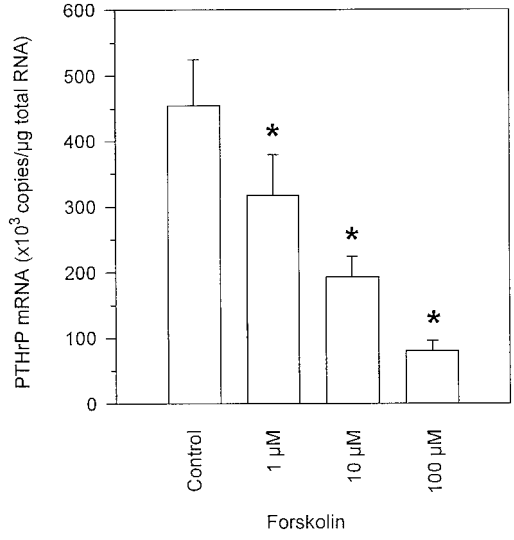

FIG. 2. PTHrP mRNA in MCF-7 breast cancer cells after incubation with 1–100 μM forskolin for 4 h ($n = 4$). *$P < 0.05$ when compared with the control group.

cells has been shown to be influenced both by systemic hormones [23, 24], and substances present in the microenvironment such as cytokines and growth factors in a paracrine or autocrine manner [25]. *In vivo*, PTHrP also seems to have paracrine/autocrine effects and it is an important local mediator of skeletal metastasis due to breast cancer. This effect of PTHrP does not always cause hypercalcemia or elevated levels of PTHrP in blood [10].

The present study shows that sCT- or forskolin-induced stimulation of adenylate cyclase was associated with suppressed PTHrP gene expression. This suppressive effect was both dependent on concentration and incubation time. Our observation that sCT

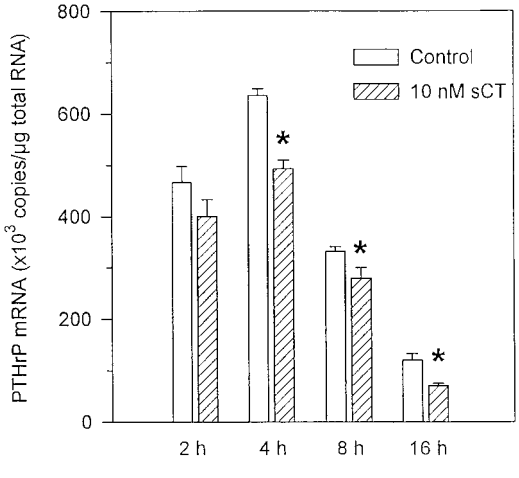

FIG. 3. PTHrP mRNA in MCF-7 breast cancer cells after incubation with 10 nM salmon calcitonin for different time periods ($n = 6$). *$P < 0.05$ when compared with their respective control groups.

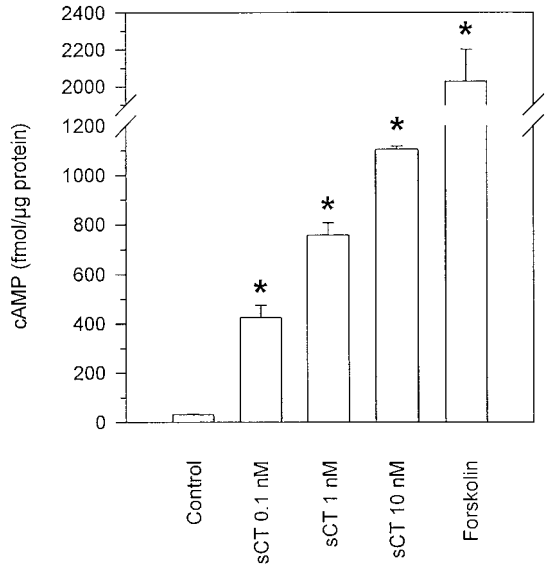

FIG. 4. Intracellular cAMP levels in MCF-7 breast cancer cells after incubation with 0.1–10 nM salmon calcitonin for 15 min ($n = 6$ in all occasions). *$P < 0.05$ when compared with the control group.

decreased the PTHrP levels in the conditioned medium by the same pattern as the mRNA further confirms the suppressive effect of sCT. PTHrP mRNA levels were measured by RT-PCR with quantification of the PCR product by time-resolved lanthanide fluorometry, which allows detection of small changes in mRNA expression. In the present study our method [19] was further improved by the introduction of an additional internal standard, β-actin cDNA, as a control of both reverse transcription and RNA degradation.

The interactions between CT and PTHrP have not been thoroughly studied. According to the conventional regulatory loop one would assume that CT should antagonize the PTH-like effects of PTHrP and have an inhibitory effect on PTHrP expression and secretion. However, an opposite effect of CT has been reported in a lung cancer cell (BEN cells) [14, 26]. In other malignant tissues such as esophagus carcinoma and submandilar carcinoma cells no effect of calcitonin on PTHrP expression has been observed [27].

These conflicting results are not surprising since CT receptors are functionally heterogeneous and they can exist in several different isoforms [28]. These receptors are coupled to multiple trimeric G proteins, thereby activating several signaling proteins, including protein kinase C, cAMP-dependent protein kinase A and also calcium/calmodulin-dependent protein kinase [29]. Moreover, the CT-activated signaling pathway is not only tissue and cell type specific, but also sometimes even cell cycle specific [30]. Therefore, the ultimate effect of CT is determined by the dominant signaling mechanism during the phase of the experiment.

The inhibitory effect of sCT on PTHrP in MCF-7 breast cancer cells observed in our study at both mRNA and protein levels might indicate a tissue or cell specific activity of CT. Further studies are required to determine whether this suppressive effect can be found also in other breast cancer cell lines and *in vivo*.

Calcitonin is sometimes used in the treatment of breast cancer patients with hyperclacemia. If the inhibitory effect on PTHrP expression is true also *in vivo*, calcitonin treatment could have potentially beneficial effects on both suppression of HHM and prevention of skeletal metastases.

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