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THE ROLE OF PARATHYROID HORMONE-RELATED PROTEIN (PTHrP) IN THE PHYSIOLOGY OF THE ADULT MAMMARY GLAND

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

by

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* * * * *

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ABSTRACT

Parathyroid hormone-related protein (PTHrP) was discovered in 1987 as the agent responsible for hypercalcemia associated with certain malignancies, including breast cancer. Attempts to characterize the pathogenesis of this syndrome revealed that PTHrP is produced by many normal fetal and adult tissues, frequently with co-expression of the PTH/PTHrP receptor suggesting a paracrine or autocrine function for PTHrP in normal physiology. Within the mammary gland, transgenic mice have demonstrated that PTHrP is required for the morphogenesis of the developing gland. However, the functions of PTHrP during lactation and breast cancer are not well understood. The overall goal of these studies was to investigate the role of PTHrP within the late pregnant and lactating mammary gland. Bovine PTHrP was cloned and a single PTHrP mRNA transcript encoding the 141 amino acid isoform of PTHrP was demonstrated in the bovine mammary gland. Within the lactating mammary gland, PTHrP was expressed in the alveolar epithelial cells, whereas PTH/PTHrP receptors were present in alveolar epithelial cells, myoepithelial cells, and stromal fibroblasts. These data suggest both paracrine and autocrine signaling mechanisms are mediated by PTHrP in the lactating mammary gland. Furthermore, exogenous PTHrP increased intracellular cAMP levels in mammary stromal fibroblasts, demonstrating that the PTH/PTHrP receptor in these cells is biologically active.
Administration of 5,6-dichloro-benzimidazole riboside (DRB) or cycloheximide slowed the decline in steady-state PTHrP mRNA levels in cultured rat mammary organoids, indicating that PTHrP mRNA stability is an important factor regulating PTHrP production and that PTHrP mRNA degradation may be mediated by a labile protein in mammary tissue.
Dedicated to Mom, with love.

Me
ACKNOWLEDGEMENTS

I would like to thank my best friend and mother, Frances, who’s contributions to this body of work have been the key to the success of this endeavor. For always being there, for sharing your wisdom and limitless vision, and for simply loving me; thank you. I hope you like what we have done.

It goes without saying that numerous others have contributed to this work. Thanks to all those with whom I have shared intellectual and scientific discussions; who have provided technical expertise in data collection, data analysis and data presentation; and who have provided supplies and access to equipment.

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CHAPTER 1

Cloning of Bovine Parathyroid Hormone-Related Protein (PTHrP) cDNA and Expression of PTHrP mRNA in the Bovine Mammary Gland

Abstract

Parathyroid hormone-related protein (PTHrP) produced by the mammary gland has been postulated to have multiple functions in both the mother and neonate. In humans, alternative 3' - mRNA splicing and endoproteolytic processing result in multiple bioactive PTHrP peptides. Multiple PTHrP peptides have also been reported in bovine milk. To investigate the source of molecular heterogeneity of PTHrP in bovine milk, bovine PTHrP was cloned from a bovine brain cDNA library, sequenced and used to characterize the mammary PTHrP transcript. A 1065 base pair clone for bovine PTHrP (bP1) was isolated from a brain cDNA library. The clone (bP1) contained the entire coding sequence of PTHrP and 61 and 473 nucleotides of the 5'- and 3'-untranslated regions (UTRs), respectively. The predicted amino acid sequence of bovine PTHrP was 72-92% homologous to the sequences of chicken, rat, mouse, human, and canine PTHrP with the highest sequence divergence present in the C-terminal region of the peptide. The 5'- and 3'-UTRs of bovine brain PTHrP have a high degree of homology to exons 4 and 9 of human PTHrP, respectively. PTHrP was expressed as a single 1200 nt mRNA transcript in lactating bovine mammary tissue.
RT-PCR using region-specific oligonucleotide primers derived from bPl demonstrated that PTHrP mRNA transcripts in bovine brain and lactating mammary gland utilize the same 5' - and 3'- UTRs. Expression of PTHrP mRNA was localized to secretory and ductular epithelial cells within the lactating mammary gland using in situ hybridization. Expression of PTHrP mRNA was demonstrated in the mammary gland during late pregnancy and throughout lactation in cows.

Introduction

Parathyroid hormone-related protein (PTHrP) was initially identified as a product of human and animal tumors associated with humoral hypercalcemia of malignancy (1, 2). Subsequently, the expression of PTHrP mRNA and protein have been reported in other neoplasms as well as many normal fetal and adult tissues in both animals and humans, including the mammary gland (3, 4, 5).

PTHrP is produced in the mammary gland during late pregnancy and lactation and is postulated to have multiple effects in both the mammary gland as well as the neonate, including regulation of calcium transport, relaxation of smooth muscle, and regulation of cell growth and differentiation (6, 7). In addition, overexpression of PTHrP in the mammary gland of transgenic mice impaired branching morphogenesis of ducts during prepubertal development as well as lobuloalveolar development during pregnancy supporting a physiological role for PTHrP in mammary gland morphogenesis (8).
PTHrP has been demonstrated in the milk of multiple species including human (9, 10), bovine (9, 11), rat (4), and goat (12) where concentrations of PTHrP in milk are 1,000-10,000 fold higher than the concentrations observed in maternal blood. Multiple bioactive PTHrP peptides have been identified in both human and bovine milk (9). In humans, these peptides are thought to arise from endoproteolytic processing of PTHrP as well as alternative splicing of PTHrP mRNA which results in 3 different isoforms of PTHrP that differ in their C-terminus. Three different PTHrP mRNA transcripts have been demonstrated in human breast tumors (13) and more recently, in normal lactating human mammary tissue (14). The biologic significance of the different PTHrP isoforms has not been established. Alternative splicing of PTHrP mRNA transcripts has not been demonstrated in the rat, mouse, or chicken. The expression of PTHrP mRNA in the mammary tissue of bovines has not been previously reported.

In the present study, we describe the cloning and sequencing of bovine PTHrP cDNA. The cDNA sequence and the predicted amino acid sequence for bovine PTHrP were compared with genomic DNA and deduced amino acid sequences of multiple species. The bovine mammary PTHrP mRNA transcript was characterized and the temporal expression of PTHrP mRNA within the bovine mammary gland was investigated. In addition, in situ hybridization was used to localize PTHrP mRNA expression within lactating bovine mammary tissue.
Materials and Methods

Cloning and sequencing of bovine PTHrP cDNA: A 434-bp bovine PTHrP cDNA probe was generated by RT-PCR from total RNA isolated from bovine hypothalamus using primers derived from the coding and 3'-UTR of human PTHrP corresponding to amino acids 6-12 (5' - CAG CTC CTC CAT GAC AAG GGG - 3') of the mature peptide and nucleotides 6-25 (5' - CCT TGG AAG GTC TCT GCT GA - 3') in the 3'-UTR, for the sense and antisense primers, respectively. The bovine PTHrP cDNA probe was labeled with α-[32P]-dATP using a random primer labeling kit (Gibco-BRL, Gaithersburg, MD) and used to screen approximately 5 x 10^5 plaques of a bovine brain cDNA library prepared in phage 8 UNI-ZAP™XR (Stratagene Cloning Systems, La Jolla, CA). Hybridization was carried out overnight at 42°C in 2X PIPES, 50% formamide, 0.5% SDS, and salmon sperm DNA at 100 μg/ml. After washing at 60°C in 0.1X SSC and 0.1% SDS for 1 hour, the membranes were exposed to x-ray film overnight at -80°C. Putative clones were selected and rescreened until individual plaques were isolated. Plasmid DNA (pBluescript) from positive clones was isolated and a positive clone with an insert of greater than 1 kb (designated bP1) was further characterized. Both strands were sequenced using an Applied Biosystems Model 373A DNA Sequencer. (Sequencing was performed by the University of Michigan Sequencing Center, Ann Arbor, MI.) Internal oligonucleotide primers for stepwise sequencing were synthesized commercially (Integrated Data Technologies, Coralville, IA).
**Tissue Procurement:** Normal mammary tissue from seventeen heifers and cows was obtained within 15 minutes of euthanasia, frozen in liquid nitrogen, and stored at -80°C until RNA isolation. Samples included normal mammary tissue from non-pregnant heifers, heifers pregnant with their first calf, and adult cows in various parities and stages of lactation as listed in Table 1.1.

**RNA isolation:** Total RNA was extracted using RNAzol B (Tel-Test, Inc., Friendswood, TX) or Trizol (Gibco-BRL), quantitated, and stored in Formazol (Molecular Research Center, Inc., Cincinnati, OH) at -80°C. Messenger RNA was isolated from total RNA by incubating with oligo-dT cellulose, and washing and eluting retained poly (A)+ RNA using the Fast Track mRNA isolation kit (InVitrogen, San Diego, CA).

**Northern Blot Hybridization:** Total RNA or poly (A)+ mRNA (10-30 µg) was separated by gel electrophoresis in a 1.2% agarose/2.2 M formaldehyde gel and transferred overnight to a nylon membrane (Duralon UV, Stratagene) in 20X SSC. The membrane was incubated in prehybridization solution [50% formamide, 5X SSC, 10 mM Tris-HCl (pH 7.5), 125 µg/ml salmon sperm DNA, 1X Denhardt’s solution, 0.2% SDS and 1% background quencher (Molecular Research Center, Inc.)] for 2 hours at 42°C. [32P]-dATP- labeled 434-bp bovine PTHrP cDNA probe was added and hybridization was continued overnight at 42°C. The membrane was washed in 1X SSC and 0.1% SDS at 50°C for 1 hour and exposed to x-ray film overnight at -80°C. Total RNA (10 µg) isolated from a canine
apocrine adenocarcinoma associated with humoral hypercalcemia of malignancy was used as a positive control for PTHrP (15).

**Oligonucleotide Primers:** Oligonucleotides used to detect PTHrP mRNA expression in bovine mammary tissue by RT-PCR were derived from bP1 and the published sequence of human PTHrP (Fig. 1.1). To amplify the coding region of PTHrP, one of two primer pairs was used; primers P1 (5' - CAg TGG AGC GTC GcG GTG TTC - 3'; mismatches shown in lower case) and P11 (5' - GTA cGT CTC CAC CTT GTT - 3'; mismatches shown in lower case) which amplify a fragment of 320 bp or primers P1 and P57 (5' - GAG TTG AGC TCC AGC GAT - 3') which amplify a fragment of 499 bp. Region-specific oligonucleotide primers derived from bP1 were used to investigate the 5'- and 3'- UTRs of PTHrP transcribed in lactating bovine mammary tissue. Primer P84 (5' - GGT TGG AGT AGC TGA TTC - 3') corresponding to nucleotides within the exon 4-like region of bP1 and primer P82 (5' - GCA CCG AGT AGC TCA GCA GG - 3') were used to amplify a 108 bp fragment containing part of the 5' - UTR. Primer P56 (5' - AGA GCA GCC ACT GAA GAC - 3') and primer P54 (5' - CAG CAG CAC CAA GAT ACA - 3') corresponding to nucleotides within the exon 9-like region of bP1 amplify a 456 bp fragment containing part of the 3' - UTR. Sense (5' - GAG ACG TTC AAC ACT CCT GC - 3') and antisense (5' - GAG CTT CTC CTT GAT GTC AC - 3') primers for bovine β-actin were based upon the published sequence of bovine lymphocyte β-actin cDNA (GenBank # K00622) and amplify.
a fragment of 276 bp. All oligonucleotides were synthesized commercially (Integrated DNA Technologies).

Reverse-Transcription/Polymerase Chain Reaction (RT-PCR): Total RNA (1 µg) was reverse transcribed with 100U Moloney Murine Leukemia Virus reverse transcriptase (Gibco-BRL) at 37°C for 30 min. in buffer containing 0.5 mM of each dNTP and 2.5 µM random hexamers. Complimentary DNA was amplified using 1U of Taq DNA Polymerase (Gibco-BRL) in buffer containing 2 mM MgCl₂ and 50 pmoles of each oligonucleotide primer. The PCR amplification was conducted for 40 cycles in a thermocycler (Perkin Elmer Cetus 9600 Gene Amp PCR System) with a thermocycle profile of denaturation at 94°C for 20 sec, primer annealing for 20 sec at 60°C and primer extension at 72°C for 1 min. PCR products were separated and visualized in a 2% agarose gel containing ethidium bromide. Total RNA isolated from lactating rat mammary tissue amplified with oligonucleotide primers P1 and P11 was used as a positive control for PTHrP. RT-PCR reactions omitting reverse-transcriptase or RNA were used as negative controls.

Southern Blot Analysis: DNA from agarose gels was transferred to a nylon membrane (Duralon UV, Stratagene) by capillary action, hybridized overnight at 42°C in hybridization solution containing 500,000 cpm of either α-[³²P]-dATP- labeled 434-bp or 1066 bp bovine PTHrP cDNA probe, washed at 37°C, and exposed to X-ray film overnight at -80°C (16).
Synthesis of riboprobes: (Riboprobe synthesis was completed by Vicky Kartsogiannis and Dr. Hong Zhou, Dept. of Medicine, University of Melbourne, Melbourne, Australia.) A PTHrP riboprobe corresponding to the common coding exon of human PTHrP (exon 6) was used to localize the PTHrP mRNA transcript in lactating bovine mammary tissue by in situ hybridization (17). A 422 bp genomic PCR fragment corresponding to nucleotides +102 through +524 of the human PTHrP cDNA sequence (1) was subcloned into the EcoRI site of pAM-19 (Amersham International, Buckinghamshire, U.K.) in both orientations. The plasmid was linearized with BamHI and transcribed by T7 RNA polymerase into antisense and sense riboprobes. The riboprobes were labeled with digoxigenin during RNA transcription using an RNA labeling kit (Boehringer Mannheim, Mannheim GmbH, Germany) according to the manufacturer’s instructions.

In situ Hybridization: (In situ hybridization was completed by Vicky Kartsogiannis and Dr. Hong Zhou, Dept. of Medicine, University of Melbourne, Melbourne, Australia.) In situ hybridization was performed on formalin-fixed, paraffin-embedded lactating bovine mammary tissue as described (17). Nonhybridized RNA was removed by treatment with 25 μg/ml of RNase A (Sigma) in 2X SSC. To block nonspecific binding of antibody used for detection, sections were incubated for 30 minutes in PBS containing 30% normal rabbit serum, 3% bovine serum albumin, and 0.1% Triton X-100 (Sigma). Hybridized probe was detected with alkaline phosphatase-coupled antidigoxigenin antibody (Boehringer Mannheim). Sections were counterstained with nuclear fast red. Sections used as negative
controls were incubated either with sense riboprobe or treated with 100 μg/ml of RNase A in 2X SSC (2 hours, 37°C) prior to prehybridization.

**Results**

**Bovine PTHrP cDNA sequence and interspecies comparison:** The bovine brain PTHrP cDNA clone (bPl) was 1065 nucleotides (Fig. 1.1) and contained a 61 nucleotide 5'-UTR, an open reading frame of 531 nucleotides encoding a protein of 177 amino acids, and a 473 nucleotide 3'-UTR. The first 39 nt of the 5'-UTR of bPl were 80% homologous to the 3'-region of human PTHrP exon 4, suggesting that in the brain, bovine PTHrP is transcribed from a P3-like (downstream TATA) promoter (18, 19, 20). Nucleotides 40-61 in the 5'-UTR of bPl were 82% homologous to the untranslated sequences present in human PTHrP exon 5.

Comparison of the deduced bovine PTHrP amino acid sequence with sequences for human (21), mouse (18), rat (21), canine (22), and chicken (23) PTHrP predicted a 36 amino acid prepro-region and a 141 amino acid mature bovine PTHrP peptide. Bovine PTHrP retains all putative di-, tri-, and multibasic proteolytic processing sites and the putative amidation sequences present at positions 86 and 94 that have been reported for human PTHrP (24). Bovine PTHrP was 92%, 90%, 87%, 85% and 72% homologous to canine, human, mouse, rat, and chicken PTHrP, respectively (Fig. 1.2). Interspecies comparison of amino acids 1-111, which comprise the N-terminal and mid-region of
PTHrP, demonstrated few conservative substitutions. Most of the amino acid sequence divergence was in the C-terminal region of PTHrP (amino acids 112-141).

The 3'-UTR of bPl was 473 nucleotides and had 96% sequence homology with the 3'-UTR of exon 9 of the human PTHrP gene including corresponding positions for the polyadenylation signal and multiple AUUUA instability sequences (18, 20).

Identification and characterization of the bovine mammary PTHrP transcript: A Northern blot (Fig. 1.3, A) containing 10-30 μg of poly (A)+ mRNA derived from lactating bovine mammary tissue was probed with α-[32P]-dATP-labeled bovine cDNA (434 bp) corresponding to the coding sequence of PTHrP. A single 1200 nt mRNA transcript for PTHrP was detected in lactating bovine mammary tissue.

RT-PCR using region-specific oligonucleotide primers derived from the 5'- and 3'-UTRs of bPl were used to characterize the 5'- and 3'-regions of mammary gland PTHrP mRNA. RT-PCR of total RNA from lactating bovine mammary tissue using each of the oligonucleotide primer pairs depicted in Fig. 1.3, B, amplified cDNAs of the expected sizes. Primers P1/P11 (Lane 2, Fig. 1.3, B) and P1/P57 (Lane 3, Fig. 1.3, B) amplified the expected 320 bp and 499 bp coding region fragments. Fragments of 108 bp and 456 bp were observed with primers P82/P84 (Lane 4, Fig. 1.3, B) and P54/P56 (Lane 5, Fig. 1.3, B), respectively, indicating that the same 5'- and 3'-UTRs used in brain PTHrP mRNA are also used in mammary PTHrP mRNA. Southern blot analysis using α-[32P]-dATP-labeled bPl (full-length bovine brain PTHrP cDNA) probe demonstrated that these PCR
products were PTHrP-specific (data not shown). The origin of the additional PTHrP-specific cDNAs amplified in lanes 4 (300 bp) and 5 (400 bp) is uncertain, but may be due to PCR artifact or amplification of genomic DNA. Amplification of human PTHrP genomic DNA with primers P82/P84 would produce a fragment of 270 bp encoding portions of exons 4 and 5 as well as the intervening intron. The additional 300 bp PCR product observed in lane 4 using primers P82 and P84 is consistent with an interpretation of genomic contamination although the genomic sequence of bovine PTHrP has not been reported. PCR products were not observed in RT-PCR reactions lacking RNA or reverse transcriptase.

**Expression of PTHrP mRNA in the bovine mammary gland:** The temporal expression of PTHrP mRNA in the bovine mammary gland was examined in non-pregnant heifers, pregnant heifers and cows during various stages of lactation using RT-PCR and Southern blot analysis (Fig. 1.4). PTHrP mRNA was expressed in the bovine mammary gland during late pregnancy and throughout lactation. Amplification of either a 320 or 499 bp PTHrP-specific RT-PCR product was detected in 50% (1 of 2) of heifers in the latter third of their first pregnancy, 88% (7 of 8) of cows during early lactation (< 100 days), 100% (2 of 2) of cows during mid-lactation (100-200 days), and 100% (2 of 2) of cows during late lactation (>200 days). In the single cow examined during the immediate pre-parturient non-lactating period, PTHrP-specific RT-PCR products were not observed on agarose gels, but a faint signal was detected on Southern blots. Cow # 17 was 4.5 months pregnant with an embryo.
transfer calf and had not lactated within the previous year. PTHrP mRNA was not detected in the mammary gland of this cow. In addition, PTHrP-specific RT-PCR products were not observed in the mammary gland of a non-pregnant, non-lactating heifer (1/1). Amplification of β-actin mRNA by RT-PCR confirmed the quality of the mRNA within the samples. The temporal expression of PTHrP mRNA in the bovine mammary gland detected in this study is summarized in Table 1.1.

*In situ* hybridization was used to localize the PTHrP mRNA transcript within the lactating bovine mammary gland. Positive staining for PTHrP mRNA was observed within most mammary alveolar and ductular epithelial cells (Fig. 1.5). (*In situ* hybridization was completed by Vicky Kartsogiannis and Dr. Hong Zhou, Dept. of Medicine, University of Melbourne, Melbourne, Australia.)

**Discussion**

PTHrP produced by the mammary gland has been postulated to have important functions in both the mother and neonate (7). Multiple PTHrP peptides as well as intact PTHrP have been detected in both bovine and human milk (9), however the biologic significance of these different molecular forms is uncertain. In other organs such as bone, different portions of the PTHrP molecule have been reported to cause different or opposing biologic effects (25, 26, 27). The PTHrP peptides observed in milk could arise from multiple mechanisms including post-translational endoproteolytic processing, extracellular proteolytic degradation, and alternative mRNA splicing.
The identification of a single 1200 nucleotide PTHrP mRNA transcript in lactating bovine mammary tissue which utilizes the same 5' and 3' UTRs as the PTHrP transcript in brain suggests that bovine PTHrP has a similar genomic organization as the rat and mouse (Fig 1.6). The mouse PTHrP gene contains at least 4 exons that are homologous to human exon 4 (5' - UTR), exon 5 (5' - UTR and preproregion of PTHrP), exon 6 (the coding sequence of mature PTHrP), and exon 9 (3' - UTR) (19, 20, 21). In the rat, PTHrP is transcribed from a P3-like promoter located upstream from exon 4 and contains a single 3' exon resulting in a 1500 nt PTHrP transcript which has been detected in many tissues (4, 20, 21). In contrast, the human PTHrP gene is more complex consisting of 9 exons with three promoters and alternative splicing of mRNA which result in multiple PTHrP mRNA transcripts (19). Direct evidence for alternatively spliced mRNAs in tissues from non-human species has not been reported. Our data suggest that the molecular heterogeneity of PTHrP observed in bovine milk does not result from alternative mRNA splicing.

The predicted sequence of mature bovine PTHrP (1-111) differs from human, rat, and mouse, and canine PTHrP by 3 or 4 amino acid residues. These substitutions occur at amino acids 49, 66, 92, and 98 and involve changes in either serine or basic amino acids which could potentially affect glycosylation or proteolytic cleavage of PTHrP. An O-linked glycosylated form of PTHrP has been detected in human keratinocytes but localization and significance of the glycosylation have not been determined (28). Amino acids 1-111 of chicken PTHrP contain multiple amino acid differences compared to mammalian PTHrPs; the biological significance of these changes are unknown.
The carboxy-terminal portion of PTHrP (112-141) is not highly conserved among reported species. The C-terminal region of bovine PTHrP is 30%, 43%, 50%, 67%, and 70% homologous to chicken, rat, mouse, human, and canine PTHrP, respectively. The biological function of the C-terminal PTHrP peptide is not clearly understood and may have divergent functions in different species.

PTHrP mRNA expression was demonstrated in the bovine mammary gland during late pregnancy and throughout lactation. The regulation of PTHrP mRNA expression in bovine mammary tissue is unknown. Although prolactin is not required for the maintenance of lactation in cows, serum prolactin is increased during lactation after milking and prolactin has been demonstrated to increase production of PTHrP in bovine primary mammary cell cultures (29). These results are similar to findings in rats in vivo where transcription of PTHrP mRNA in the lactating rat mammary gland was stimulated by prolactin and was dependent upon suckling for continued expression (30). It is possible that milking regulates PTHrP expression by the mammary gland of ruminants since milking increased the concentration of PTHrP detected in the milk of goats (31). Lack of milking for an extended period before tissue sampling may explain why PTHrP mRNA expression was not detected in 1 of 12 lactating cows in this study.

In summary, we have described the cloning and sequencing of bovine PTHrP and examined the expression of PTHrP mRNA within the bovine mammary gland. Analysis of the cDNA and amino acid sequences of bovine PTHrP and characterization of the mammary PTHrP mRNA transcript suggest that the multiple PTHrP fragments observed
in bovine milk are the result of endoproteolytic processing or extracellular degradation of PTHrP, rather than alternative splicing of the PTHrP mRNA transcript. PTHrP synthesized within epithelial cells in the lactating mammary gland is the likely source of PTHrP observed within milk. The expression of PTHrP mRNA observed in the mammary gland of cows in this study is consistent with a physiologic role for PTHrP in both mammary development and lactation.

References


17. Zhou, H., Choong, P., McCarthy, R., Chou, S. T., Martin, T. J., & Ng, K. W. 1994 In situ hybridization to show sequential expression of osteoblast gene markers


30. Thiede, M. A. 1989 The mRNA encoding a parathyroid hormone-like peptide is produced in mammary tissue in response to elevations in serum prolactin. *Molecular Endocrinology* 3 1443-1447.

Figure 1.1. Nucleotide sequence of bovine brain PTHrP cDNA (bP1). The predicted coding region is shown in capital letters. The 5' and 3' untranslated regions are shown in lower-case letters. Deduced amino acids are shown below their corresponding codons. The sequence encoding the mature protein begins at nucleotide 170. The AUUUA instability sequences in the 3' UTR are underlined. The polyadenylation signal is boxed. The location of the sense and antisense oligonucleotide primers used for RT-PCR are indicated by arrows.
Figure 1.1. Nucleotide sequence of bovine brain PTHrP cDNA (bP1).
Figure 1.2. Comparison of amino acid sequences of mature parathyroid hormone-related protein (PTHrP) from bovine, human, rat, mouse, canine, and chicken. The top line represents the deduced amino acid sequence of bovine PTHrP derived from a bovine brain cDNA library. Conserved residues in human, rat, mouse, canine, and chicken are designated by a dot. Gaps due to additions or deletions of residues are designated by a dash.
<table>
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<th></th>
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<th>mouse</th>
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<td>(50)</td>
<td>(50)</td>
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Figure 1.2. Comparison of amino acid sequences
Figure 1.3. Characterization of bovine mammary PTHrP mRNA transcript: Panel A: Northern Blot Analysis: Lane 1, positive control, contains 10 μg of total RNA isolated from a canine apocrine adenocarcinoma. Lanes 2, 3, and 4, contain 10, 20, and 30 μg, respectively, of poly (A)* mRNA isolated from lactating bovine mammary tissue (Cow #9). Membrane was probed with α-[32P]-dATP-labeled 434 bp bovine PTHrP cDNA. Panel B: Region-specific RT-PCR: Lane 1, 123 bp DNA ladder. Lanes 2-5, RT-PCR of 1 μg of total mammary RNA from cow #9 using primers P1 and P11 (coding region)(lane 2), P1 and P57 (coding region)(lane 3), P82 and P84 (5' UTR)(lane 4), and P54 and P56 (3' UTR)(lane 5).
Figure 1.3. Characterization of bovine mammary PTHrP mRNA transcript
Figure 1.4. Expression of PTHrP mRNA in bovine mammary tissue *in vivo*. RT-PCR and Southern blot analysis for PTHrP: Total RNA was isolated from bovine mammary tissue at various stages of pregnancy and lactation, reverse-transcribed and amplified with primers for PTHrP or β-actin (lane numbers correspond to the cow ID #s from Table 1, page 26). PCR products were transferred to a nylon membrane by capillary action and probed with 500,000 cpm of α-[32P]-dATP-labeled 434 bp bovine PTHrP cDNA. RT-PCR products for β-actin were visualized in agarose gels stained with ethidium bromide. Expression of RT-PCR products for β-actin confirmed the quality of RNA within the sample. Total RNA isolated from lactating rat mammary tissue amplified with oligonucleotide primers P1 and P11 was used as a positive control for PTHrP. RT-PCR of total RNA from lactating rat mammary tissue without using reverse-transcriptase was used as negative control.
Figure 1.4. Expression of PTHrP mRNA in bovine mammary tissue *in vivo*. 
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<td>2</td>
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<td>P</td>
</tr>
<tr>
<td>3</td>
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<td>3</td>
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<td>9</td>
<td>3 days postpartum, lactating</td>
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N= negative, P = positive.

Table 1.1. Animals used in this study.
Figure 1.5. Cellular localization of PTHrP mRNA within lactating bovine mammary tissue using in situ hybridization. Formalin-fixed, paraffin-embedded sections of mammary tissue from a lactating cow (cow #9) were hybridized with 1-4 ng/μl of digoxigenin-labeled PTHrP riboprobe for 16-18 hours at 42°C. Nonhybridized RNA was removed by treatment with RNase A. Hybridized probe was detected with alkaline-phosphatase coupled anti-digoxigenin antibody. Sections were counterstained with nuclear fast red. Top panel: Positive staining for PTHrP was detected in most alveolar and ductular epithelial cells. Bottom panel: Negative control. Section was treated with RNase A prior to prehybridization. (In situ hybridization was completed by Vicky Kartsogiannis and Dr. Hong Zhou, Dept. of Medicine, University of Melbourne, Melbourne, Australia.)
Figure 1.5. Cellular localization of PTHrP mRNA within lactating bovine mammary tissue
Figure 1.6. Comparison of the organization of the human and mouse PTHrP genes with the cDNA sequences of bovine and rat PTHrP. Untranslated regions are shown in cross hatched boxes, the preproregion is shown in white, and the coding sequence of the mature peptide is shown in black. Numbers correspond to exons of the human PTHrP gene. Arrows indicate observed and predicted mRNA splicing patterns.
Figure 1.6. Comparison of the organization of the human and mouse PTHrP genes with the cDNA sequences of bovine and rat PTHrP.
CHAPTER 2

Expression of PTHrP and the PTH/PTHrP Receptor in Purified Alveolar Epithelial Cells, Myoepithelial Cells, and Stromal Fibroblasts Derived from the Lactating Rat Mammary Gland

Abstract

Parathyroid hormone-related protein (PTHrP) is produced by the lactating mammary gland and secreted into the milk; however, the function of PTHrP during lactation is unknown. Since messenger RNA for both PTHrP and the PTH/PTHrP receptor have been demonstrated within mammary tissue, a paracrine or autocrine function for PTHrP has been proposed. To investigate this hypothesis in lactating tissue, the expression of PTHrP and the PTH/PTHrP receptor were examined in purified subpopulations of cells derived from lactating rat mammary glands. Subpopulations of stromal, myoepithelial, and alveolar epithelial cells were isolated from mammary tissue using enzymatic digestion and immunomagnetic purification. Isolated cells were phenotypically characterized by immunohistochemistry and ultrastructural morphology. The purity of the separated alveolar and myoepithelial cells was assessed ultrastructurally and ranged from 91-96%. Messenger RNA and protein expression of PTHrP and the PTH/PTHrP receptor were examined using
reverse transcription polymerase chain reaction and immunohistochemistry and western blot analysis, respectively. PTHrP mRNA and protein were expressed in alveolar epithelial cells and stromal fibroblasts, whereas PTH/PTHrP receptor mRNA and protein were expressed in all three cell types. The expression patterns for PTHrP and the PTH/PTHrP receptor support an autocrine or paracrine function for PTHrP in alveolar epithelial cells and stromal fibroblasts, and a paracrine function for PTHrP in myoepithelial cells in the rat mammary gland during lactation.

Introduction

Parathyroid hormone-related protein (PTHrP) is produced in the normal mammary gland during embryologic and prepubertal development as well as late pregnancy and throughout lactation (1, 2, 3, 4). Results from studies in transgenic mice indicate that carefully regulated levels of PTHrP are required for proper mammary growth and development since PTHrP null mice lack structures derived from mammary epithelium, and overexpression of PTHrP by mammary myoepithelial cells results in mammary gland hypoplasia. Furthermore, the effects of PTHrP are mediated by the PTH/PTHrP receptor since PTHrP and PTH/PTHrP receptor null mice exhibit the same mammary phenotype (1, 2).

Less is known about the role of PTHrP produced by the lactating mammary gland; however, multiple functions for PTHrP have been proposed in both the mother and neonate. These include the regulation of calcium metabolism and transport, growth and
differentiation of tissues, and smooth muscle tone within the vascular system and gastrointestinal tract (5). Expression of the PTH/PTHrP receptor has been detected within rat lactating mammary tissue by northern blot analysis in our lab, suggesting a paracrine or autocrine function for PTHrP within the lactating gland. Specific physiological roles that have been postulated for PTHrP within the lactating mammary gland involve the regulation of growth and differentiation of mammary epithelial cells, transepithelial calcium transport into the milk, and milk ejection by modulating myoepithelial cell function (5).

To further investigate the paracrine and autocrine functions of PTHrP in the mammary gland, the cell-to-cell interactions involved in PTHrP signaling need to be defined. During embryologic development of the mammary gland, PTHrP production has been demonstrated in immature mammary epithelial cells, and receptors for PTH/PTHrP have been demonstrated in primitive mesenchymal cells (1, 2, 6). These findings suggest a paracrine function for PTHrP within the mammary gland that modulates epithelial-mesenchymal interactions similar to the function of PTHrP reported in skin (1, 7). In lactating mammary tissue, PTHrP production has been localized to alveolar epithelial cells in rats and cows by in situ hybridization; however, cell-specific localization of the PTH/PTHrP receptor has not been reported (3, 8). The presence of PTH/PTHrP receptors on myoepithelial cells has been suggested since cultured primary mammary myoepithelial cells demonstrated a rise in intracellular cyclic AMP in response to exogenous PTHrP (9).

In this study, we examined the messenger RNA and protein expression of PTHrP and the PTH/PTHrP receptor in purified cell subpopulations derived from lactating rat
mammary tissue to determine which cells produce PTHrP and which express the PTH/PTHrP receptor. Our data demonstrate cell-specific expression of PTHrP and the PTH/PTHrP receptor and support both paracrine and autocrine functions for PTHrP within the lactating mammary gland.

Materials and Methods

Preparation of monodispersed rat mammary cell suspensions: Cells for these studies were obtained from lactating mammary tissue of Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.; Indianapolis, IN) and processed according to the method of Kim and Clifton (10) with modifications (Fig. 2.1). At days 4-20 postpartum, rats were euthanatized using 70% CO₂ according to an approved animal use protocol. Lactating mammary tissue was aseptically harvested and minced in chilled serum-free medium (SFM; Dulbecco’s modified Eagle’s medium (Gibco-BRL; Gaithersburg, MD), with 50 μg/ml gentamicin sulfate). Minced tissue was incubated for 1.5 hours at 150 rpm on a rotary shaker (150 rpm) at 37°C in SFM containing 2 mg/ml of collagenase (Type III), (Worthington Biochemical Corporation; Lakewood, NJ.). During the last 15 min, 3 ml of 0.05% DNase (Sigma Chemical Company; St. Louis, MO) was added per 90 mls of collagenase solution.

For the isolation of rat mammary stromal fibroblasts, the suspension was centrifuged at 100xg to pellet organoids and larger cell clumps. The supernatant containing single cells was transferred to a new tube and centrifuged at 1000xg. The pellet was washed and plated in 100 mm² culture dishes (Becton Dickson Labware; Plymouth, England) in
DMEM:F12 containing 10% FBS (Gibco-BRL), 50 μg/ml of gentamicin sulfate, and 10 μg/ml of insulin (Sigma). After two hours of incubation in 5% CO₂ at 37°C, the medium with non-attached cells was aspirated and fresh medium was added.

To isolate mammary myoepithelial and alveolar epithelial cells, a monodispersed cell suspension was produced from the cell pellet containing organoids and larger cell clumps. The pellet was resuspended in 30 ml of serum-containing medium (SM; DMEM with 10% FBS and 50 μg/ml gentamicin sulfate), washed in SM, then filtered through a 40 micron pore nylon mesh filter (Tetko, Inc.; Briarcliff Manor, NY). Retained organoids were collected in SM medium, washed with SFM, and resuspended in 10 ml of prewarmed 0.05% Trypsin-EDTA (Gibco-BRL). The suspension was placed at 37°C for 11 min on a rotary shaker. Digestion was stopped by the addition of 25 ml of SM. The cell pellet was washed in SFM, resuspended in SFM containing 0.05% DNase, and filtered through a 20 micron pore nylon mesh filter (Tetko, Inc.). Filtrate containing predominantly single cells was collected, concentrated, and quantitated with a hemocytometer.

**Immunostaining of monodispersed cells:** Monodispersed cells were resuspended in PBS containing 1% BSA to a final concentration of 2 X 10⁷ cells/ml. The monodispersed cell sample was divided, and incubated for 30 mins at 4°C with either biotinylated peanut agglutinin lectin (1.25 μg/ml), (Vector Laboratories, Inc.; Burlingame, CA), which binds to the luminal surface of alveolar epithelial cells or mouse anti-rat Thy 1 monoclonal
antibody (8 μg/ml), (Harlan Sera-Lab LTD; Sussex, England) which binds to the surface of myoepithelial cells (11, 12). Samples were washed in PBS containing 1% BSA before immunomagnetic purification.

**Immunomagnetic purification of cell subpopulations:** Immunologically-labeled alveolar and myoepithelial cells were separated magnetically from unlabeled cells by the addition of magnetic beads conjugated to either streptavidin or sheep anti-mouse immunoglobulin G, respectively. Magnetic beads (Dynabeads: Dynal, Oslo, Norway) were washed twice in PBS-0.1% BSA and incubated with the labeled monodispersed cells at a concentration of 1 X 10^7 beads/ml. Incubations were continued for 30 min at 4°C. Alveolar epithelial and myoepithelial cells were isolated by applying an external magnet to retain labeled cells while the unlabeled cells present in solution were aspirated. Samples were washed in this fashion three times with PBS-0.1% BSA. Samples of separated cells were plated on glass slides for immunohistochemistry, fixed in 3% glutaraldehyde for ultrastructural examination, or aliquoted in Trizol (Gibco-BRL) for RNA isolation.

**Immunohistochemistry:** Immunomagnetically-purified myoepithelial and alveolar epithelial cells and cultured rat mammary stromal cells (passages 1-4) were fixed in acetone at -20°C. Slides were equilibrated to room temperature, rehydrated in PBS and incubated with 0.2 N HCl or 1.5% H₂O₂ to inactivate endogenous alkaline phosphatase or peroxidases, respectively. Slides were blocked with PBS containing 5% horse serum, then
incubated with primary antibody in 1% horse serum overnight at 4°C. The following primary antibodies were used: monoclonal mouse anti-human cytokeratin (1:50), (MNF116; DAKO; Carpinteria, CA), monoclonal mouse anti-vimentin (1:50), (DAKO), monoclonal mouse anti-α-actin (1:100), (Boehringer Mannheim Corporation; Indianapolis, IN), polyclonal rabbit anti-PTHLP (PTHrP), (1:100), (Oncogene Research Products; Cambridge, MA), and polyclonal rabbit anti-PTH receptor (PTH/PTHrP receptor), (1:100), (BABCO; Richmond, CA). Slides were then washed with PBS and incubated with secondary antibody in 1% horse serum for 1 hr at room temperature. An alkaline-phosphatase conjugated rat anti-mouse IgG was used as the secondary antibody for cytokeratin, vimentin, and α-actin (1:1000), (Pierce; Rockford, IL). After incubation with secondary antibody, slides were washed in PBS, incubated overnight in BCIP/NBT solution substrate (AMRESCO; Solon, OH), and counterstained with nuclear fast red. A biotinylated goat anti-rabbit IgG was used as the secondary antibody for PTHrP and the PTH/PTHrP receptor (1:1000), (Zymed Laboratories, Inc.; San Francisco, CA). After incubation with the secondary antibody, slides were washed in PBS, incubated with avidin-biotin complex (Pierce), followed by diaminobenzidine for color development (Research Genetics; Huntsville, AL), and counterstained with hematoxylin. Immunohistochemical staining with omission of the primary antibody was used as the negative control for cytokeratin, smooth muscle actin and vimentin. The negative control for PTHrP and the PTH/PTHrP receptor consisted of immunohistochemical staining with primary antibody that had been preincubated with 10⁻⁶ M antigen.
Electron microscopy: (Samples for electron microscopy were prepared by Ms. Evelyn Handley, Dept. of Veterinary Biosciences, OSU, Columbus, OH.) Separated cells were fixed in 3% glutaraldehyde, rinsed in cacodylate buffer, post-fixed in osmium tetroxide, and dehydrated in a graded series of ethanol followed by propylene oxide. Samples were embedded in a Medcast resin, sectioned on an LKB ultramicrotome at 700 angstroms, and stained with uranyl acetate/lead citrate. Samples were examined using a Phillips 300 electron microscope. Cells were classified and quantitated to assess the purity of each sample.

Isolation of total RNA: Total RNA was extracted from the samples using Trizol according to the manufacturers instructions. Samples were quantitated and stored at -80°C.

Oligonucleotide Primers: Oligonucleotides used to detect PTHrP mRNA expression were derived from the published sequence of human PTHrP (Genbank M24350, M24351). Primers P1 (5’-CAG TGG AGC GTC GCG GTG TTC-3’) and P11 (5’- GTA CGT CTC CAC CTT GTT-3’) amplify a 320 bp fragment consisting of portions of exons 5 and 6 of PTHrP. Oligonucleotides used to detect PTH/PTHrP receptor mRNA expression were derived from the published sequence of the human PTH/PTHrP receptor (Genbank U22404, U22405). Primers P20 (5’- ACC AAT GAG ACT CGT GAA CG-3’) and P21 (5’- AAG GAC AGG AAC AGG TGC ATG-3’) amplify a 167 bp fragment of the PTH/PTHrP receptor consisting of exons 5, 6, and 7. Primers for rat β-casein and α-actin
were derived from the published sequences (Genbank M11175, M11178; X06801). P25 (5' - CCA TGA AGG TCT TCA TCC - 3') and P26 (5' - TTG CTG CAC AAG GTA CTG - 3') amplify a fragment of 577 bp consisting of exons 2-7 of rat β-casein. Primers P17 (5' - TGT GAA GAG GAA GAC AGC - 3') and P18 (5' - GCC TGA ATA GCC ACA TAC - 3') amplify a 415 bp fragment of rat α-actin. All primers were synthesized commercially (Integrated DNA Technologies; Coralville, IA)

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR):** Total RNA (1 µg) was reverse transcribed with 100 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) at 37°C for 30 min in buffer containing 0.5 mM of each dNTP and 2.5 µM random hexamers. Complimentary DNA was amplified using 1 U of Taq DNA polymerase (Gibco-BRL) in buffer containing 2 mM MgCl₂ and 50 pmol of each oligonucleotide primer. The PCR amplification was conducted for 40 cycles in a thermocycler (Perkin-Elmer-Cetus 9600 Gene Amp PCR System; Perkin-Elmer-Cetus, Norwalk, CT) with a thermocycle profile of denaturation at 94°C for 20 s, primer annealing at 60°C for 20 s, and primer extension at 72°C for 1 min. PCR products were separated and visualized in a 2% agarose gel containing ethidium bromide. Total RNA from lactating rat mammary tissue amplified with primers PL/PLI and P20/P21 were used as positive controls for PTHrP and the PTH/PTHrP receptor, respectively. RT-PCR reactions omitting reverse transcriptase or RNA were used as negative controls.
Southern Blot Analysis: DNA from agarose gels was transferred to a nylon membrane (Duralon UV, Stratagene, La Jolla, CA) by capillary action, hybridized overnight at 42°C in hybridization solution containing 500,000 cpm of either α-[32P]dATP-labeled 434 bp bovine PTHrP probe (corresponding to amino acids 6-141 of the mature protein and the first 25 nucleotides of the 3' UTR) or 674 bp PstI fragment of the cloned human PTH/PTHrP receptor (HKrK) encoding portions of exons 2-8, washed at 37°C, and exposed to X-ray film overnight at -80°C.

Protein Isolation and Western Blot Analysis: Total protein was isolated from cultured mammary stromal fibroblasts by scraping cells from tissue culture plates with cold TBS (100 mM Tris pH 7.5, 0.9% NaCl), pelleting and resuspending cells in an equal volume of RIPA buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% IGEPAL CA-630, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA) containing COMPLETE protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Cells were shaken in 1.5 ml tubes for 30 mins at 4°C to extract proteins, and repelleted. The supernatant was stored at -20°C. Total protein was isolated from mammary tissue by grinding tissue under liquid nitrogen and resuspending in an equal volume of RIPA buffer containing protease inhibitors. The tissue suspension was homogenized and processed as for proteins isolated from cultured cells. Total proteins (100 µg per sample) were separated on 15% SDS-polyacrylamide gel and blotted to nitrocellulose filters. Filters were blocked for 30 min in 0.1% Tween 20, 1% powdered milk in TBS then incubated for 30 mins with polyclonal rabbit anti-PTHLP
(PTHrP), (1:50) (Oncogene Research Products; Cambridge, MA). Blots were washed and sequentially incubated with secondary antibody (biotinylated goat anti-rabbit IgG; Zymed) (1:500), avidin-biotin complex, and diaminobenzidine. The midregion antigenic peptide (NH$_2$ - RGRRRSGTPVDKNEKSEC -Ac) to which the PTHrP antibody was generated was used as a positive control. (This peptide was synthesized by the University of Michigan Protein Facility, Ann Arbor, MI.)

Results

Characterization of Separated Cells: Cells isolated by immunomagnetic purification with peanut agglutinin exhibited histologic, immunohistochemical and ultrastructural features consistent with alveolar epithelial cells. Histologically, these cells were large and contained round nuclei with abundant cytoplasm and discrete intracytoplasmic fat globules. Purified cells were immunohistochemically positive for cytokeratin and negative for smooth muscle actin and vimentin, consistent with the epithelial origin of these cells (Figs. 2.2 & 2.3). Ultrastructural morphology of the alveolar epithelial cells was typical of cells actively synthesizing milk. These cells had large round nuclei with abundant cytoplasm, mitochondria and rough endoplasmic reticulum. In addition, electron dense casein micelles within secretory vesicles and multiple large lipid droplets were present within the cytosol (Fig. 2.4, A).

Cells isolated by immunomagnetic purification with anti-Thy 1 antibody exhibited histologic, immunohistochemical and ultrastructural features consistent with myoepithelial
cells. Histologically, myoepithelial cells contained small ovoid nuclei with moderate to abundant cytoplasm and multiple cytoplasmic processes. Purified cells were immunohistochemically positive for smooth muscle actin and cytokeratin, but negative for vimentin (Figs. 2.2 & 2.3). Ultrastructurally, these cells contained ovoid or convoluted nuclei, finely granular, moderately electron dense cytoplasm with cytoplasmic processes, a paucity of cytoplasmic organelles, and scattered intracytoplasmic filaments with focal electron dense material typical of contractile cells (Fig. 2.4, B).

Cultured mammary stromal fibroblasts were flattened, polygonal to spindle-shaped cells with ovoid nuclei and moderate to abundant cytoplasm with many cellular processes. Cultured cells were immunohistochemically positive for vimentin, but negative for smooth muscle actin and cytokeratin, consistent with a fibroblastic origin for these cells (Figs. 2.2 & 2.3).

**Purity of Separated Cell Subpopulations:** The purity of the alveolar epithelial and myoepithelial cells was assessed by electron microscopy and ranged from 91-96%. At least five hundred intact nucleated cells were counted per sample. Cells were classified as either alveolar epithelial or myoepithelial, or indeterminate cells based on the following criteria: 1) Cells were classified as alveolar epithelial cells if they had round nuclei with abundant cytoplasm containing many mitochondria, rough endoplasmic reticulum, casein micelles and intracytoplasmic lipid droplets; 2) cells were classified as myoepithelial cells if they had ovoid or convoluted nuclei with finely granular cytoplasm, cytoplasmic processes, and
intracytoplasmic filaments with focal electron dense material; and 3) cells that did not fit either of these former criteria we classified as indeterminate cells. Degenerate cells characterized by cell shrinkage, condensation of chromatin, dilation of cytoplasmic organelles, and an increased number of lysosomes were also classified as indeterminate cells and generally comprised less than 3% of the cells counted.

Detection of PTHrP and the PTH/PTHrP Receptor in Isolated Cell Subpopulations: Protein expression of PTHrP and the PTH/PTHrP receptor was examined in the separated cells by immunohistochemistry (Fig. 2.3). Alveolar epithelial cells stained positively for PTHrP diffusely throughout the cytoplasm. A significantly less intense positive signal for PTHrP was observed in the cytoplasm of cultured mammary stromal fibroblasts. The positive immunohistochemical signal for PTHrP observed within alveolar epithelial cells and stromal fibroblasts was lost when the primary antibody was preincubated with soluble antigen, demonstrating the specificity of the PTHrP antibody. Positive immunohistochemical staining for PTHrP was not detected within myoepithelial cells.

The expression of PTHrP protein in the cultured stromal fibroblasts also was examined by western blot analysis (Fig. 2.5). Multiple immunopositive bands for PTHrP were observed in the fibroblasts, including two bands of 30 kDa and 27 kDa apparent molecular weight, and a fainter band of 22 kDa molecular weight. These bands are approximately the expected size of full length rat PTHrP, and its presumed proteolytic products, taking into account that the mid-region PTHrP peptide used as a positive control
ran at a much higher apparent molecular weight (10 kDa) than its calculated molecular weight of 2553 Da. In both human and bovine milk, PTHrP peptides have been shown to migrate with higher apparent molecular weights than the theoretical relative mobilities ($M_r$). PTHrP peptides of 33-34 kDa, 21-27 kDa, and 13 kDa have been demonstrated by western blot analysis of human and bovine milk (13). The apparent molecular weights of immunopositive PTHrP proteins observed in mammary tissue extracts differed from that observed in the fibroblasts. These changes were attributed to a difference in proteolytic processing within whole mammary tissue. Expression of PTHrP protein observed by western blot analysis in these cells is consistent with the expression observed by immunohistochemistry.

Protein expression of the PTH/PTHrP receptor was observed in all three cell types but was most strongly positive in the alveolar epithelial and myoepithelial cells (Fig. 2.3). There was enhanced staining for the receptor along the margins of the alveolar and myoepithelial cells. A less intense positive immunohistochemical signal for the PTH/PTHrP receptor was detected in cultured mammary stromal fibroblasts. In addition, fibroblasts frequently exhibited a well demarcated, eccentric, perinuclear positive stain for the PTH/PTHrP receptor, suggesting vesicular localization of the PTH/PTHrP receptor within the cell. Positive immunohistochemical staining for the PTH/PTHrP receptor was lost when the primary antibody was preincubated with soluble antigen, demonstrating the specificity of the PTH/PTHrP receptor antibody.
**Messenger RNA Expression of PTHrP and the PTH/PTHrP Receptor in the Isolated Cell Subpopulations:** Messenger RNA expression of PTHrP and the PTH/PTHrP receptor were examined in the isolated rat mammary cells using the reverse transcription-polymerase chain reaction and Southern blot analysis (Figs. 2.6, A and 2.6, B). A cDNA fragment of 320 bp was amplified by RT-PCR in the alveolar epithelial cells and stromal fibroblasts, consistent with the expression of PTHrP mRNA in these cells. A very faint 320 bp band of amplified cDNA was observed in the myoepithelial cells, indicating that a small amount of PTHrP mRNA was present within this sample. The specificity of the 320 bp fragment for PTHrP was confirmed by Southern blot analysis (Fig. 2.6, B).

Expression of PTHrP mRNA in the alveolar epithelial cells, the predominant cell type present during lactation, was decreased compared to PTHrP mRNA expression observed in total RNA isolated from frozen lactating mammary tissue (Fig. 2.6, A; lane 2), indicating that some degradation of the PTHrP mRNA transcript had occurred during the 9-hour cell separation and purification procedure. The PTHrP mRNA detected within the myoepithelial cells may represent a relatively small amount of PTHrP production by these cells. However, the lack of positive immunohistochemical staining for PTHrP in the myoepithelial cells suggests that the PTHrP cDNA observed within this sample is due to the small percentage of alveolar epithelial cells present, as was observed ultrastructurally and demonstrated by the positive β-casein mRNA expression.

Expression of PTH/PTHrP receptor mRNA was observed within all cell types, with the highest expression present in the myoepithelial cells and stromal fibroblasts (Fig. 2.6,
A). The specificity of the 167 bp cDNA band for the PTH/PTHrP receptor was confirmed by Southern blot analysis (Fig. 2.6, B). Messenger RNA for PTHrP, the PTH/PTHrP receptor, β-casein, or α-actin was not amplified in RT-PCR samples that lacked the reverse transcriptase.

Discussion

In this study we have investigated the hypothesis that PTHrP produced by the lactating mammary gland is an autocrine or paracrine regulator of mammary function. By examining the expression of PTHrP and the PTH/PTHrP receptor in purified subpopulations of lactating mammary cells, we were able to localize PTHrP and PTH/PTHrP receptor expression to specific cell types. Messenger RNA and protein expression for both PTHrP and the PTH/PTHrP receptor were concordant. The results of this study demonstrated PTHrP production by rat mammary stromal fibroblasts during lactation in addition to that previously reported for alveolar epithelial cells. This is the first report of cell-specific PTH/PTHrP receptor localization in lactating mammary cells. Our results demonstrate PTH/PTHrP receptor expression in alveolar epithelial cells, myoepithelial cells, and stromal fibroblasts. The patterns of expression of PTHrP and the PTH/PTHrP receptor observed support both paracrine and autocrine functions for PTHrP in the mammary gland during lactation.

The expression of PTHrP mRNA and protein by alveolar epithelial cells has been reported during late pregnancy and lactation in rats, and during lactation in cows (3, 8). The
results of this study also demonstrated PTHrP mRNA expression and protein production in lactating rat alveolar epithelial cells. In addition, we have demonstrated expression of the PTH/PTHrP receptor in these cells, suggesting an autocrine function for PTHrP in alveolar epithelial cells during lactation. Coexpression of PTHrP and the PTH/PTHrP receptor is common in many human breast cancers and epithelial breast cancer cell lines (14, 15, 16). In MCF-7 (15) and 8701-BC (16) breast cancer cells, PTHrP has been reported to regulate growth and invasion in an autocrine fashion. The effect of PTHrP on growth and differentiation or calcium transport in lactating mammary alveolar epithelial cells has not been investigated.

Production of PTHrP by myoepithelial cells has been demonstrated in vitro (17). A human mammary myoepithelial cell line (Hs578Bst) derived from non-lactating breast tissue has been reported to produce PTHrP in culture (17). In addition, immunohistochemical expression of PTHrP has been demonstrated within normal myoepithelial cells during prepubertal development of the mammary gland and within adult non-lactating mammary tissue, however, the PTHrP detected may not have been produced by myoepithelial cells (2, 18). The limits of resolution of in situ hybridization have precluded the use of this technique to examine messenger RNA expression for PTHrP in myoepithelial cells during lactation (3, 8). Our results suggest that, during lactation, myoepithelial cells do not produce significant levels of PTHrP in vivo. A very faint band of PTHrP cDNA was observed in the ethidium bromide-stained gel after RT-PCR on RNA isolated from purified myoepithelial cells. The data suggest that the PTHrP observed within
this sample was due to the small percentage of alveolar epithelial cells present. However, it is possible that myoepithelial cells express a low level of PTHrP which is below the level of sensitivity for immunohistochemical detection. Alternatively, expression of PTHrP in mammary myoepithelial cells may vary with stage of development and differentiation.

The presence of PTH/PTHrP receptors on myoepithelial cells has been suggested in cultured cells. Both primary rat mammary myoepithelial cells isolated from lactating tissue and Hs578Bst cells produce a rise in intracellular cAMP in response to exogenous PTHrP(1-34) (9, 17). The results of this study which demonstrate PTH/PTHrP receptor mRNA and protein expression in myoepithelial cells are consistent with this hypothesis. Our data suggest a paracrine function for PTHrP on myoepithelial cells during lactation. In Hs578Bst cells, PTHrP has been reported to modulate oxytocin signaling (17). A similar effect may occur in myoepithelial cells during lactation supporting the role of PTHrP in modulating milk ejection.

Expression of the PTH/PTHrP receptor has been previously reported in dermal fibroblasts (19) and mesenchymal cells within the mammary stroma during embryonic development (1). In this report, we have demonstrated both PTHrP and PTH/PTHrP receptor expression in stromal fibroblasts isolated from the lactating mammary gland, suggesting both paracrine and autocrine functions for PTHrP on these fibroblasts. However, the in vivo significance of the expression of PTHrP observed within these stromal fibroblasts is uncertain as expression of PTHrP mRNA within these cells by in situ
hybridization has not been reported (3) and serum induction of PTHrP expression has been demonstrated in cultured cells (20).

Within the developing mammary gland, intercellular signaling between embryonic epithelial cells which produce PTHrP and the primitive mesenchymal cells which express PTH/PTHrP receptors is required for mammary development (1, 6). In skin, PTHrP is also believed to be an important regulator of epithelial-mesenchymal interactions involving PTHrP and IGF-1 signaling between keratinocytes and dermal fibroblasts (7). As in the developing mammary gland and skin, PTHrP produced by alveolar epithelial cells during lactation may act through PTH/PTHrP receptors located on stromal fibroblasts to modulate growth and differentiation of alveolar epithelial cells or remodeling of the extracellular matrix.

In summary, we have demonstrated cell-specific expression of PTHrP and the PTH/PTHrP receptor in cells isolated from lactating mammary tissue. The data support the hypothesis that PTHrP functions as an autocrine or paracrine regulator in the lactating mammary gland. The results of this study suggest possible cell to cell interactions between alveolar epithelial cells, myoepithelial cells, and stromal fibroblasts which are regulated by PTHrP during lactation.

References


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Figure 2.1. Flow chart indicating the procedure used for isolation of mammary alveolar epithelial cells, myoepithelial cells and stromal fibroblasts.
Figure 2.1. Flow chart indicating the procedure used for isolation of mammary alveolar epithelial cells, myoepithelial cells and stromal fibroblasts.
Figure 2.2. Immunohistochemical characterization of immunomagnetically-purified mammary alveolar epithelial cells (AEC), myoepithelial cells (MEC), and stromal fibroblasts (FBL). Immunohistochemistry for cytokeratin (KER; panels A-C)), smooth muscle actin (SMA; panels D & E)), and vimentin (VIM; panel F), are shown. Positive immunostaining is indicated by a dark grey color. Cytokeratin is expressed in AEC and MEC. Smooth muscle actin is expressed in MEC. Vimentin is expressed in FBL. Negative controls (CON) consisted of immunohistochemical staining with omission of the anti-cytokeratin (panel G), anti-smooth muscle actin (panel H) and anti-vimentin (panel I) primary antibodies. The spherical objects in the AEC and MEC are the magnetic beads. Magnification 665X (AEC and MEC), 250X (FBL).
Figure 2.2. Immunohistochemical characterization of immunomagnetically-purified mammary alveolar epithelial cells (AEC), myoepithelial cells (MEC), and stromal fibroblasts (FBL).
Figure 2.3. Immunohistochemical expression of PTHrP and the PTH/PTHrP receptor in immunomagnetically-purified mammary alveolar epithelial cells (AEC), myoepithelial cells (MEC), and stromal fibroblasts (FBL). Immunohistochemistry for PTHrP (panels A-C) and the PTH/PTHrP (panels D-F) are shown. Positive immunostaining is indicated by a dark grey color. PTHrP protein is demonstrated in AEC and FBL. Expression of PTH/PTHrP receptor protein is observed in AEC, MEC, and FBL. Perinuclear staining for the PTH/PTHrP receptor in stromal fibroblasts is indicated by the arrowheads. Negative controls (CON) consisted of immunohistochemical staining with primary antibody pre-incubated with PTHrP antigen (panel G) or PTH/PTHrP receptor antigen (panels H and I). The spherical objects in the AEC and MEC are the magnetic beads used for cell purification. Magnification 665X (AEC and MEC), 250X (FBL).
Figure 2.3. Immunohistochemical expression of PTHrP and the PTH/PTHrP receptor in immunomagnetically-purified mammary alveolar epithelial cells (AEC), myoepithelial cells (MEC), and stromal fibroblasts (FBL).
Figure 2.4. Transmission electron micrographs of immunomagnetically-purified cells.  
4A: Alveolar epithelial cells. Note casein micelles (arrowheads) within secretory vesicles (sv). Cells contain abundant rough endoplasmic reticulum (er) and mitochondria (mt). 4B: Myoepithelial cells. Cells contain cytoplasmic filaments with focal electron dense material (fil) characteristic of contractile cells and numerous cytoplasmic processes (cp). (Scale bars = 1.6 μm).
Figure 2.4. Transmission electron micrographs of immunomagnetically-purified cells.
Figure 2.5. Western blot analysis for PTHrP protein within the cultured stromal fibroblasts. Lane 1: Mid-region PTHrP peptide (5 µg). Lane 2: Mammary tissue (100 µg of total protein). Lane 3: Stromal fibroblasts (100 µg of total protein). The apparent molecular size of the immunoreactive PTHrP protein bands observed are indicated (kDa).
Figure 2.5. Western blot analysis for PTHrP protein within the cultured stromal fibroblasts.
Figure 2.6. Messenger RNA expression of PTHrP and the PTH/PTHrP receptor in purified alveolar epithelial cells (AEC), myoepithelial cells (MEC) and mammary stromal fibroblasts (FBL). 6A: Ethidium bromide-stained agarose gel of RT-PCR for PTHrP (320 bp), PTH/PTHrP receptor (167 bp), β-casein (577 bp) and α-actin (415 bp): Lane 1: 123 bp DNA ladder, Lanes 2-3: Lactating mammary tissue, Lanes 4-5: Immunomagnetically-purified AEC, Lanes 6-7: Immunomagnetically-purified MEC, Lane 8-9: Cultured FBL. In lanes 3, 5, 7, and 9, the reverse transcriptase was omitted from the reaction. 6B: Southern blot analysis of RT-PCR for PTHrP and the PTH/PTHrP receptor in lactating mammary tissue, AEC, MEC and FBL. Lane 1: Positive control, lactating mammary tissue. Lane 2: Negative control, lactating mammary tissue with omission of reverse transcriptase. Lane 3: AEC. Lane 4: MEC. Lane 5: Cultured FBL.
Figure 2.6. Messenger RNA expression of PTHrP and the PTH/PTHrP receptor in purified alveolar epithelial cells (AEC), myoepithelial cells (MEC) and mammary stromal fibroblasts (FBL).
CHAPTER 3

Characterization of the Parathyroid Hormone/Parathyroid Hormone-Related Protein (PTH/PTHrP) Receptor in Mammary Stromal Fibroblasts

Abstract

Type I PTH/PTHrP receptors with paracrine or autocrine functions have been identified in many tissues. Within the mammary gland, PTHrP produced by epithelial cells binds to PTH/PTHrP receptors on stromal cells to regulate the morphogenesis of the developing gland. During lactation PTHrP PTH/PTHrP receptors have been demonstrated in alveolar epithelial cells, myoepithelial cells, and stromal fibroblasts, but the function of PTHrP during lactation is not known. In this study, the expression and biological activity of the PTH/PTHrP receptor was investigated in cultured mammary stromal fibroblasts isolated from the mammary tissue of rats in late pregnancy (day 18-21). Cultured fibroblasts grew as elongated spindle-shaped cells that were positive for vimentin and smooth muscle actin, and negative for cytokeratin and desmin, consistent with a myofibroblastic phenotype. Fibroblasts expressed a single 2.4 kb mRNA transcript for the PTH/PTHrP receptor by northern blot analysis. Specific binding of moniodinated [Tyr^{36}]-hPTHrP(1-36)-NH_{2} was not detected in the fibroblasts despite expression of the PTH/PTHrP receptor protein in these cells, suggesting a low level of expression of the
receptor at the cell surface. However, fibroblasts responded to exogenous PThrp(1-34) or bPTlh(1-34) with a dose-dependent increase in intracellular cAMP (maximal response; 3-4 fold increase at 1 μM). The cAMP response to PThrp(1-34) could be attenuated by preincubation of cells with the PTH/PTHrp receptor antagonist bPTlh(7-34) (1 μM). Exogenous PThrp(1-34) (1-1000 nM) and bPTlh(1-34) (10 nM) had no effect on intracellular calcium in cultured fibroblasts. However, a 3-4 fold increase in intracellular calcium was observed in these cells in response to oxytocin (0.2 IU), suggesting that the fibroblasts produced the Gaq proteins required for activation of phospholipase C. Alternative splicing of the 5'-untranslated region and the amino-terminal coding sequence of the PTH/PTHrp receptor was not observed by RT-PCR. These data suggest that mammary stromal fibroblasts in late pregnancy express low levels of the type I PTH/PTHrp receptor on the cell surface and that the biological effects of PThrp(1-34) binding to these receptors are mediated through adenylate cyclase and not phospholipase C. Furthermore, the low levels of surface expression and the selective activation of the adenylate cyclase pathway upon agonist binding are not the result of alternative 5'-splicing of the PTH/PTHrp receptor.

**Introduction**

PTHrp was originally discovered as the agent responsible for humoral hypercalcemia of malignancy (1). In this paraneoplastic syndrome, PTHrp produced by certain neoplasms circulates in the bloodstream and mimics the effects of PTH by binding
to classic PTH receptors in bone and kidney resulting in hypercalcemia. The amino-terminal sequence and structural homology of PTHrP and PTH allows both molecules to interact with the PTH/PTHrP receptor. Expression of the PTH/PTHrP receptor is observed in a wide variety of tissues frequently with local co-expression of PTHrP suggesting a paracrine or autocrine role for PTHrP in normal physiology (2).

Deciphering the role of PTHrP in normal physiology has been difficult because of the pleiotropic effects of PTHrP in different tissues. These varying effects are due, in part, to the prohormone nature of the PTHrP molecule where different functions have been attributed to the amino-terminal, midregion, and carboxyl-terminal peptides of PTHrP, each mediated by one or more distinct receptors (3-10). In addition, tissue-specific variations in signal transduction have been observed when amino-terminal PTHrP binds to the type I PTH/PTHrP receptor, indicating cell-specific regulation of receptor activation and post-receptor signal transduction mechanisms. The type I PTH/PTHrP receptor expressed in bone and kidney is a G-protein coupled receptor which is activated with equal potency by PTH and PTHrP. Upon binding of agonist, this receptor interacts with adenylyl cyclase and phospholipase C to increase intracellular cAMP and calcium. However, type I PTH/PTHrP receptors in vascular smooth muscle selectively utilize cAMP as an intracellular second messenger (11). The molecular basis for the selective activation of signal transduction pathways observed for the type I PTH/PTHrP receptor is not well understood, but has generally been attributed to different downstream interactions of the type I PTH/PTHrP receptor with G-proteins. Furthermore, the level of type I PTH/PTHrP receptor expression
may be one mechanism which regulates these interactions. The phospholipase C response to type I PTH/PTHrP receptor activation has been reported to be correlated to the number of receptors expressed at the cell surface, whereas the adenylate cyclase response was unaffected by changes in the number of cell surface type I PTH/PTHrP receptors (12). In addition, alternative splicing of the 5'-untranslated exons and exons encoding the amino-terminus of the type I PTH/PTHrP receptor have been observed in bone and kidney (13-15). Furthermore, these changes have resulted in reduced receptor expression at the cell surface and reduced biological activity (14, 15).

Within the developing mammary gland, PTHrP produced by epithelial cells binds to type I PTH/PTHrP receptors in stromal cells to regulate normal mammary ductal morphogenesis and lobuloalveolar development during pregnancy (16-19). A similar epithelial-mesenchymal paracrine loop that regulates growth has been reported in neonatal skin between keratinocytes and dermal fibroblasts (20). Recently, type I PTH/PTHrP receptor expression has been localized to alveolar epithelial cells, myoepithelial cells, and stromal fibroblasts in cells isolated from lactating mammary tissue suggesting epithelial-epithelial as well as epithelial-mesenchymal interactions which are mediated by PTHrP during lactation (21). The function of PTHrP during lactation has not been clearly established, but it is likely that PTHrP also regulates growth and functional differentiation within the lactating gland.
The purpose of this study was to investigate the mRNA and protein expression of the type I PTH/PTHrP receptor and characterize the signal transduction mechanisms used by this receptor in fibroblasts isolated from the rat mammary gland during late pregnancy.

Materials and Methods

Isolation of Primary Stromal Fibroblasts: Primary mammary stromal fibroblasts were isolated from rat mammary tissue as previously described (21). Briefly, mammary tissue from late pregnant (day 18-21) Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.; Indianapolis, IN) was aseptically harvested and minced in chilled serum-free medium (SFM; Dulbecco’s modified Eagle’s medium (Gibco-BRL; Gaithersburg, MD), with 50 μg/ml gentamicin sulfate). Minced tissue was incubated for 1.5 hr on a rotary shaker (150 rpm) at 37°C in SFM containing 2 mg/ml of collagenase (Type III), (Worthington Biochemical Corporation; Lakewood, NJ). Mammary stromal fibroblasts were isolated by centrifuging the suspension at 100xg to pellet organoids and larger cell clumps. The supernatant containing single cells was transferred to a new tube and centrifuged at 1000xg. The pellet was washed and plated in 100 mm² culture dishes (Becton Dickson Labware; Plymouth, England) in DMEM:F12 containing 10% FBS (Gibco-BRL), 50 μg/ml of gentamicin sulfate, and 10 μg/ml of ovine insulin (Sigma). After two hours of incubation in 5% CO₂ at 37°C, the medium with non-attached cells was aspirated and fresh medium was added.
Protein Isolation: Total protein was isolated from cultured mammary stromal fibroblasts (passages 2-6) by scraping cells off tissue culture plates with cold TBS (100 mM Tris pH 7.5, 0.9% NaCl), pelleting and resuspending cells in an equal volume of RIPA buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% IGEPAL CA-630, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA) containing COMPLETE protease inhibitors (Boehringer Mannheim, Indianapolis, IN). Cells were shaken in 1.5 ml tubes for 30 min at 4°C to extract proteins and repelleted. The supernatant was stored at -20°C. Total protein was isolated from whole tissue by grinding tissue under liquid nitrogen and resuspending in an equal volume of RIPA buffer containing protease inhibitors. The tissue suspension was homogenized and processed as for proteins isolated from cultured cells.

Western Blotting: Total proteins (100 μg per sample) were separated on 10-15% SDS-polyacrylamide gels and blotted to nitrocellulose filters. Filters were blocked for 30 min in 0.1% Tween 20, 1% powdered milk in TBS then incubated for 30 min with primary antibody. The following primary antibodies were used: monoclonal mouse anti-pancytokeratin (1:100) (MNF116; DAKO; Carpinteria, CA), monoclonal mouse anti-pancytokeratin (1:100) (C-11; Sigma; St. Louis, MO), monoclonal mouse anti-cytokeratin 14 (1:100) (CKB1; Sigma), monoclonal mouse anti-vimentin (1:500) (DAKO), monoclonal mouse anti-α-actin (1:1000) (Boehringer Mannheim, Indianapolis, IN), monoclonal mouse anti-desmin (1:100) (DE-U-10; SIGMA) and polyclonal rabbit anti-PTH receptor (PTH/PTHrP receptor) (1:100) (BABCO; Richmond, CA). Blots were washed 3 times in
blocking buffer, then incubated for 30 min with secondary antibody. An alkaline-phosphatase conjugated rat anti-mouse IgG (1:500-1000) (Pierce; Rockford, IL) was used as the secondary antibody for the pan-cytokeratins, vimentin, desmin, and α-actin. Blots were subsequently washed and incubated in BCIP/NBT solution substrate (AMRESCO; Solon, OH) for color development. A biotinylated goat anti-rabbit IgG (1:500-1000) (Zymed Laboratories, Inc.; San Francisco, CA) was used as the secondary antibody for the PTH/PTHrP receptor and cytokeratin 14. Blots were subsequently washed and incubated for 30 min with avidin-biotin complex (Pierce) followed by diaminobenzidine for color development.

Receptor Binding Assay: [Tyr$^{38}$]-hPTHrP(1-36)-NH$_2$ was radioiodinated and purified by HPLC as previously described (22). Binding assays were carried out with confluent cells in 24-well plates (ROS 17/2.8 cells) and 12-well plates (mammary stromal fibroblasts; passages 2-6). Culture medium was removed, and cells were incubated for 1 hr at room temperature in binding assay buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM CaCl$_2$, 5 mM KCl, 0.5% BSA and 5% FBS) containing radioiodinated PTHrP(1-36)NH$_2$ (0.1 nM) in the absence or presence of unlabeled PTHrP(1-34) (0.01-1000 nM). Cells were washed twice with binding buffer, solubilized with 500 µl of 0.5 N NaOH, and cell-associated radioactivity was determined. Cell numbers were determined by trypsinization of adjacent wells and counting the cells on a hemocytometer.
**Adenylyl Cyclase Stimulation Assay:** Stimulation assays were carried out with confluent cells in 24-well plates (ROS 17/2.8 cells) and 12-well plates (cultured mammary stromal fibroblasts; passages 2-6). Culture medium was removed and cells were incubated in calcium and magnesium-free HBSS containing 1% BSA and 1 mM isobutylmethylxanthine for 10 min at 37°C. The incubation was continued for 15 min in the absence or presence of exogenous PTHrP(1-34) or bPTH(1-34) (0.01-1000 nM). Forskolin at 10 μM was used as a positive control. Culture medium was then aspirated and 250-350 μl of 5% ice-cold perchloric acid was added. The plates were frozen overnight at -20°C. Cell numbers were determined by trypsinization of adjacent wells and counting with a hemocytometer. After thawing, the extract was transferred to tubes and the pH was adjusted to 7.5 with 4 N KOH. (The rest of this assay was completed by Ms. Amy Koh and Dr. Laurie McCauley, Department of Periodontics, Prevention and Geriatrics, University of Michigan, Ann Arbor, MI). Samples were centrifuged to remove the precipitate and the neutralized extract was assayed for cAMP using a cAMP binding protein assay. Triplicate samples of standards or unknowns were incubated for 90 min on ice with 3H-cAMP (10,000 cpm) (ICN Radiochemicals; Irvine, CA) and cAMP binding protein sufficient to bind 40-60% of the radioactivity. Dextran-coated charcoal was added to each tube and centrifuged to remove unbound from bound cAMP-binding protein-3H-cAMP complexes. The radioactivity in the supernatant was counted with a liquid scintillation spectrophotometer and cAMP concentration calculated by the log-logit method using GraphPad Prism. The mean +/- SEM on replicate samples from multiple experiments was determined. Results were analyzed for
statistical significance by one way ANOVA with a Bonferroni post-test using GraphPad Instat.

**Intracellular Calcium Assay**: Mammary stromal fibroblasts (passages 2-6) were cultured on 2-well glass coverslips (Nalge Nunc International Corp.; Naperville, IL) until confluent. Cells were loaded with 1 μM FURA-2AM (Molecular Probes; Eugene, OR) in DMEM:F12 with 1% BSA, 1mM sodium pyruvate, and 5 mM probenecid by incubating cells for 30 min at 37°C and 5% CO₂. Loaded cells were washed twice with PBS to remove unincorporated dye. Assay medium consisting of 10 mM HEPES pH 7.2, 145 mM NaCl, 5 mM KCl, 1mM MgSO₄, 0.5 mM Na₂HPQ, 5 mM glucose, and 1 mM CaCl₂ was added. (Intracellular calcium data was collected and analyzed with the help of Mr. James Hensley, Dr. Charlene Hohl, and Dr. Ruth Altschuld, Department of Medical Biochemistry, and Dr. Susan Mallery, Department of Oral Pathology, Ohio State University, Columbus, OH.)

Fluorescence 340/380 ratios were measured in loaded cells using a Nikon inverted fluorescence microscope and the PTI filterscan fiberoptic system (Photon Technology International; Princeton, NJ) (340/380 excitation, 510 emission; 40X objective). Fluorescence ratios at baseline and in response to exogenous PTHrP(1-34)(1-1000 nM), bPTH(1-34) (10 nM), or oxytocin (0.2 IU) were determined. Fluorescence maximum and minimum ratios were determined after the addition of 10 μM ionomycin and Tris:EGTA (1M Tris-HCl pH 7.2, 0.5 M EGTA), respectively. Raw data was normalized to both the minimum and maximum and plotted using GraphPad Prism.
RNA Isolation and Northern Blot Analysis: Total RNA was extracted from cultured fibroblasts, and bone, kidney, and mammary tissue homogenates using Trizol (Gibco-BRL). Total RNA (20 μg) was separated by gel electrophoresis in a 1.2% agarose/2.2 M formaldehyde gel and transferred overnight to a nylon membrane (Duralon UV, Stratagene, La Jolla, CA) in 20X SSC. The membrane was incubated in prehybridization solution [50% formamide, 5X SSC, 10 mM Tris-HCl (pH 7.5), 125 μg/ml salmon sperm DNA, 1X Denhardt's solution, 0.2% SDS and 1% background quencher (Molecular Research Center, Inc., Cincinnati, OH)] for 2 hours at 42°C. [32P]-dATP-labeled PstI fragment (674 bp) of the cloned human PTH/PTHrP receptor (HKrK) (Gift of Dr. Laurie McCauley, University of Michigan, Ann Arbor, MI) encoding portions of exons 2-8 was added and hybridization was continued overnight at 42°C. The membrane was washed in 1X SSC and 0.1% SDS at 50°C for 1 hour. Radioactivity was measured using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Total RNA (10 μg) isolated from rat kidney was used as a positive control for the PTH/PTHrP receptor.

Oligonucleotide Primers: Oligonucleotides used to detect PTH/PTHrP receptor mRNA expression were derived from the published sequences of the human (Genbank U22404, U22405) and rat PTH/PTHrP receptors (Genbank L19475) and selected manuscripts (14, 15). Sense primers PU3 (5' - GGC CTG AAC TGG AGA CAC TT - 3') corresponding to sequences within the upstream 5'-untranslated exon, or PS (5' - GCG GCC CTA GGC GGT
GG - 3') corresponding to a region of the signal sequence and the antisense primer PAS (5' - GGC ATG GCC TTT GTG ATT GAA - 3') corresponding to sequences at the junction of exons 3 and G; were used to examine the mRNA expression of the 5'-untranslated region and exons 1 and 2 of the type I PTH/PTHrP receptor, respectively. Primers P20 (5' - ACC AAT GAG ACT CGT GAA CG - 3') and P21 (5' - AAG GAC AGG AAC AGG TGC ATG - 3') amplified a 167 bp fragment of the PTH/PTHrP receptor consisting of exons 5, 6, and 7. All primers were synthesized commercially (Integrated DNA Technologies; Coralville, IA).

Reverse Transcription-Polymerase Chain Reaction: Total RNA (1 µg) was reverse transcribed with 100U Moloney Murine Leukemia Virus reverse transcriptase (Gibco-BRL) at 37°C for 30 min in buffer containing 0.5 mM of each dNTP and 2.5 µM random hexamers. Complimentary DNA was amplified using 1U of Taq DNA Polymerase (Gibco-BRL) in buffer containing 2 mM MgCl₂ and 50 pmoles of each oligonucleotide primer. The PCR amplification was conducted for 40 cycles in a thermocycler (Perkin Elmer Cetus 9600 Gene Amp PCR System) with a thermocycle profile of denaturation at 94°C for 20 sec, primer annealing for 20 sec at 60°C and primer extension at 72°C for 1 min. PCR products were separated and visualized in a 2% agarose gel containing ethidium bromide. RT-PCR reactions omitting reverse-transcriptase were used as negative controls.
Southern Blotting: DNA from agarose gels was transferred to a nylon membrane (Duralon UV, Stratagene) by capillary action, hybridized overnight at 42°C in hybridization solution containing 500,000 cpm of an α-[³²P]-dATP-labeled 451 bp PCR fragment of the rat type I PTH/PTHrP receptor encoding exons S, E1, E2, and E3. This probe was generated by reverse transcription of total RNA isolated from rat kidney followed by PCR amplification of cDNA with primers PS and PAS. Probe was gel purified using a Qiagen gel extraction kit (Qiagen, Inc.; Chatsworth, CA). After hybridization, blots were washed at 37°C, and measured with a PhosphorImager (Molecular Dynamics, Inc.).

Results

Characterization of Cultured Mammary Stromal Fibroblasts: Mammary stromal fibroblasts isolated from the mammary glands of rats during late pregnancy grew in culture as elongated spindle-shaped cells (Fig. 3.1). The phenotype of these cells was examined by western blot analysis (Fig. 3.2). Proteins immunopositive for vimentin (62 kDa) and smooth muscle actin (37 kDa) were observed in protein extracts of cultured fibroblasts (passages 2-6) and dermis (positive vimentin control) or stomach (positive smooth muscle actin control).

Cytokeratin and desmin protein expression were not present in the cultured fibroblasts. Multiple immunopositive proteins with apparent molecular sizes of 36-48 kDa were observed with the pancytokeratin antibody (C-11) in mammary tissue (positive control). This antibody has been reported to react with antigenic epitopes for cytokeratins
4, 5, 6, 8, 10, 13, and 18. With the exceptions of cytokeratin 21 (49 kDa) and 8 (57 kDa), the specific molecular weight of rat cytokeratins is not known. Immunopositive bands for cytokeratin using either the MNF116 anti-pancytokeratin or the anti-cytokeratin 14 antibodies were not detected in cultured fibroblasts (data not shown). A protein immunopositive for desmin (59 kDa) was observed in skeletal muscle (positive control) but not the cultured fibroblasts.

These data are consistent with a myofibroblastic phenotype of the cultured cells. Expression of smooth muscle actin in these fibroblasts was likely due to culture in vitro. The differentiation of myofibroblasts from fibroblasts has been reported in cells cultured at low cell density (23). Myofibroblasts are not normally present within mammary tissue but have been observed in the desmoplastic response of invasive human breast cancers (24).

**Expression of the PTH/PTHrP Receptor in Cultured Fibroblasts:** The mRNA and protein expression of the type I PTH/PTHrP receptor in cultured fibroblasts was investigated by northern and western blot analysis, respectively (Fig. 3.3). A single 2.4 kb mRNA transcript for the type I PTH/PTHrP receptor was observed in the fibroblasts, consistent with the size previously reported for this receptor observed within other rat tissues (25).

Expression of the type I PTH/PTHrP receptor protein in cultured fibroblasts was confirmed by western blot analysis. Multiple immunopositive bands for the receptor were observed in both the fibroblasts and mammary tissue (positive control), including two proteins with molecular weights of approximately 85 kDa. An 85 kDa protein has been
identified as the PTH/PTHrP receptor by photoaffinity labeling (26). The slightly higher molecular weight form may be a phosphorylated form of the receptor. The 150 kDa immunopositive protein present in both samples, as well as the 130 kDa immunopositive protein observed in mammary tissue may represent aggregates of the type I PTH/PTHrP receptor.

Functional Properties of the PTH/PTHrP Receptor in Cultured Fibroblasts: Specific binding of [Tyr$^{36}$]-hPTHrP(1-36)-NH$_2$ was not observed in the cultured rat mammary stromal fibroblasts, but could be demonstrated in ROS 17/2.8 cells (positive control), suggesting that the surface expression of the PTH/PTHrP receptor in the fibroblasts was below the level of sensitivity of detection for this assay (data not shown).

A dose-dependent increase in intracellular cAMP was observed in the cultured fibroblasts in response to exogenous PTHrP(1-34) (0.01-1000 nM) (Fig. 3.4)(Part of this assay was completed by Ms. Amy Koh and Dr. Laurie McCauley, Department of Periodontics, Prevention, and Geriatrics, University of Michigan, Ann Arbor, MI). A maximal 3 to 4-fold increase in intracellular cAMP was observed at 1μM PTHrP(1-34). A similar increase in intracellular cAMP was also observed with bPTH(1-34) (0.1-1000 nM) (Fig. 3.5). Furthermore, preincubation of fibroblasts with the PTH/PTHrP receptor antagonist bPTH(7-34) (1 μM), attenuated the effects of PTHrP(1-34) in these cells (Fig. 3.6). These data suggest that the response of these fibroblasts to exogenous PTHrP or PTH is mediated by the type I PTH/PTHrP receptor.
Adenylyl cyclase stimulation in stromal fibroblasts in response to exogenous PTHrP(1-34) was less than the response observed in ROS 17/2.8 cells (Fig. 3.7). The maximal cAMP response to exogenous PTHrP(1-34) detected in the ROS 17/2.8 cells was approximately equivalent to the response of these cells to forskolin, a direct activator of adenylyl cyclase; whereas the maximal response to exogenous PTHrP(1-34) in the cultured fibroblasts was only 25% of the response observed with forskolin. These results are consistent with a lower expression of the type I PTH/PTHrP receptor at the cell surface in the cultured fibroblasts.

No change in intracellular calcium occurred in response to either exogenous PTHrP(1-34) (1-1000 nM) or bPTH(1-34) (10 nM) in cultured mammary stromal fibroblasts (Fig. 3.8) (This data was collected with the help of Mr. James Hensley, Dr. Charlene Hohl, and Dr. Ruth Altschuld, Department of Medical Biochemistry, and Dr. Susan Mallery, Department of Oral Pathology, Ohio State University, Columbus, OH.). However, addition of oxytocin (0.2 IU) resulted in a transient 3 to 4-fold increase in intracellular calcium in the fibroblasts, indicating that these cells were viable and contained Gaq proteins which are able to interact with phospholipase C.

Characterization of PTH/PTHrP Receptor mRNA Transcripts in Cultured Stromal Fibroblasts: The 5' -region of type I PTH/PTHrP receptor mRNA transcripts in the cultured fibroblasts was investigated by RT-PCR using exon specific primers (Figs. 3.9 and 3.10). The gene for the rat type I PTH/PTHrP receptor contains two promoters (14). Transcription
from the downstream P2 promoter is observed in most tissues. However, alternative promoter usage and alternative 5'-splicing of the type I PTH/PTHrP receptor have been demonstrated in bone and kidney (13-15). Splice variants of the type I PTH/PTHrP receptor which lack either the signal sequence or exon 1 have been associated with reduced expression of the receptor at the cell surface and reduced biological activity (14, 15).

Expression of type I PTH/PTHrP receptor mRNA was observed in mammary stromal fibroblasts, bone and kidney using primers P20 and P21 which amplify a cDNA fragment of 167 bp encoding exons 5, 6, and 7 of the receptor (Fig. 3.10, A: lanes 4, 8, 12).

Primers PU3 and PAS were used to examine promoter usage for the type I PTH/PTHrP receptor in the cultured fibroblasts. Transcription initiation from the upstream P1 promoter, which is located upstream of the 5'-untranslated exon U3, has only been reported in kidney and ovary (13, 14); however, transcription from this promoter has not been examined in mammary tissue. Messenger RNA transcripts for the type I PTH/PTHrP receptor which utilize the P1 promoter were not detected in cultured fibroblasts or bone (Fig. 3.10, A: lanes 3 and 7). Three cDNA fragments of approximately 520, 580, and 640 bp were amplified in the kidney with primers PU3 and PAS, consistent with the expected sizes of the three type I PTH/PTHrP mRNA transcripts previously reported to be initiated from the P1 promoter within the kidney (Fig. 3.10, A: lane 11)(14).

Primers PS and PAS were used to examine the 5' coding region of type I PTH/PTHrP receptor mRNA transcripts within the cultured mammary stromal fibroblasts. Splice variants of the type I PTH/PTHrP receptor which lack exon 1 have been reported in
human kidney (15). Multiple cDNA fragments were amplified with primers PS and PAS in the cultured fibroblasts, as well as in bone and kidney (Fig. 3.10, A: lanes 2, 6, 10). However, only the 451 bp cDNA fragment present in each sample was shown to be specific for the type I PTH/PTHrP receptor by Southern blot analysis (Fig. 3.10, B). A cDNA fragment of 451 bp is the expected size of amplified type I PTH/PTHrP receptor mRNA transcripts containing spliced colinear exons S, E₁, E₂, and E₃. These results indicate that alternative splicing of the 5' coding sequence of the type I PTH/PTHrP receptor does not occur in the cultured mammary fibroblasts.

**Discussion**

In this study we have investigated the expression and functional properties of the type I PTH/PTHrP receptor in cultured mammary stromal fibroblasts derived from pregnant rat mammary tissue (days 18-21 of gestation). Both type I PTH/PTHrP receptor mRNA and protein expression were demonstrated in the fibroblasts. However, our results suggest that low levels of the type I PTH/PTHrP receptor are present on the surface of the fibroblasts resulting in a small (3-4 fold) increase in cAMP upon activation of this receptor with exogenous hPTHrP(1-34). Furthermore, splice variants of the type I PTH/PTHrP receptor which lack either the signal sequence or exon 1 and could account for the low levels of surface expression that have been suggested for this receptor in these fibroblasts, were not observed. Activation of phospholipase C, indicated by an increase in intracellular calcium upon agonist binding to the type I PTH/PTHrP receptor, was not detected in the cultured
fibroblasts. Our results suggest that the type I PTH/PTHrP receptor present in rat mammary stromal fibroblasts during late pregnancy is biologically active and capable of responding to a paracrine signal mediated by PTHrP produced by mammary alveolar epithelial cells.

Multiple receptors for amino-terminal peptides of both PTH and PTHrP have been proposed. In some cases, these receptors are derived from different genes. The classic type I PTH/PTHrP receptor is present in many tissues and has been cloned and well characterized (27). Both PTH and PTHrP can bind to this receptor with equal affinity and activate both adenylate cyclase and phospholipase C. A receptor selective for the amino terminus of PTH which utilizes both adenylate cyclase and phospholipase C, has recently been cloned and characterized (5, 28). Although this receptor, the PTH2 receptor, shares 70% sequence homology to the type I PTH/PTHrP receptor, it is derived from a different gene. The PTH2 receptor has a restricted tissue distribution and is primarily located in brain and pancreas. In addition, identification of a type III PTH/PTHrP receptor which is activated by PTH and PTHrP and restricted to skin and dermal bone has been reported recently in zebrafish (29). Two other receptors for amino-terminal PTH and PTHrP peptides have been suggested based upon selective cell-specific biological responses which do not appear to be mediated by the type I PTH/PTHrP receptor, but these proteins have yet to be identified and cloned. A type II PTH/PTHrP receptor which binds both PTH and PTHrP resulting in an increase in intracellular calcium but not cAMP has been reported in keratinocytes, lymphocytes, and insulinoma cells (6). In addition, a receptor selective for
PTHrP(1-34) which utilizes cAMP as an intracellular second messenger has been reported in brain (30).

Our results indicate that the biological responses to hPTHrP(1-34) and bPTH(1-34) observed in the cultured mammary fibroblasts are mediated by type I PTH/PTHrP receptors. First, a single mRNA transcript for the PTH/PTHrP receptor was identified in these cells and RT-PCR with exon specific primers derived from the type I PTH/PTHrP genomic sequence amplified fragments of the expected size for the type I PTH/PTHrP receptor. Second, an 85 kDa protein immunopositive for the PTH/PTHrP receptor, the expected size of the type I PTH/PTHrP receptor is present in the mammary stromal fibroblasts. Third, both PTH(1-34) and PTHrP(1-34) bind to the PTH/PTHrP receptor in these cells with equal affinity producing an increase in intracellular cAMP but not calcium, unlike the proposed PTH/PTHrP receptor in keratinocytes and the PTHrP receptor in brain (6, 30).

Expression of type I PTH/PTHrP receptors have been reported previously in fibroblasts isolated from skin and periodontal ligament (31, 32). An increase in intracellular cAMP upon activation of the type I PTH/PTHrP receptor in fibroblasts is commonly reported (31, 33, 34). In dermal fibroblasts, addition of bPTH(1-34) resulted in a dose-dependent 2 to 4-fold increase in intracellular cAMP with a EC₅₀ of approximately 100 nM, and a maximal 4-fold increase observed at 1 μM (33). These data are similar to those demonstrated in the mammary fibroblasts used in this study. Like the mammary fibroblasts of this study, periodontal ligament fibroblasts did not activate phospholipase C in response
to PTH or PTHrP (32). However, in the single study that has examined intracellular calcium signaling in dermal fibroblasts, transient elevations in intracellular calcium (30-300\%) were observed after the addition of PTH or PTHrP (10-1000 nM), suggesting that calcium may also mediate PTHrP signaling in dermal fibroblasts (34).

The molecular basis for the selective activation of adenylate cyclase in the cultured mammary stromal fibroblasts is currently unknown. However, selective activation of signal transduction pathways by the type I PTH/PTHrP receptor has also been observed in other cell types. In LLC-PK1 cells transfected with the type I PTH/PTHrP receptor, agonist-induced activation of phospholipase C has been correlated with receptor density at the cell surface; whereas, the adenylate cyclase response in these cells is unaffected by changes in receptor density (12). Experimental evidence suggests that this selectivity reflects the relative affinities of the type I PTH/PTHrP receptor for the Gsα and Gaq proteins. Low levels of surface expression of the type I PTH/PTHrP receptor are commonly observed in primary and early passage cells isolated from tissue. Whether these low levels of receptor expression are due to altered synthesis, receptor-mediated endocytosis or altered processing and recycling in primary cells is unknown. Low level cell-surface and a prominent perinuclear immunopositive staining for the type I PTH/PTHrP receptor in rat fibroblasts isolated from lactating mammary tissue has been reported by our laboratory and is consistent with altered synthesis or recycling of receptors in these cells (21).

In vascular smooth muscle cells transfected with and expressing high levels of the type I PTH/PTHrP receptor, competition for the binding of G-proteins has been suggested
as the mechanism responsible for the selective utilization of adenylyl cyclase in response to PTHrP in these cells (11). A higher affinity of the Gaq proteins for other G-protein coupled receptors (endothelin and thrombin receptors) rather than the type I PTH/PTHrP receptor has been reported in these cells (11). A similar regulatory mechanism may be responsible for the selective activation of adenylate cyclase in the mammary stromal fibroblasts. It is possible that the oxytocin receptors present within these cells have a much higher affinity for the Gaq proteins than the type I PTH/PTHrP receptor. Alternatively, mutations in the specific residues of the receptor that have been shown to be important for interaction with phospholipase C may account for the inability of the mammary stromal fibroblasts to activate this signal transduction pathway. Mutations in residue 319 of the second and residues 378, 379, and 382 of the third cytoplasmic loop of the type I PTH/PTHrP receptor have been associated with an impaired phospholipase C response upon receptor activation (35, 36). The sequence of these residues in the mammary stromal fibroblast type I PTH/PTHrP receptor were not examined.

Phosphorylation of G-protein coupled receptors by downstream effectors such as protein kinase A or protein kinase C, or by G-protein-coupled receptor kinases is one mechanism of desensitizing these receptors (37, 38). The two proteins bands (85 kDa) observed by western blot analysis for the type I PTH/PTHrP receptor in the rat mammary stromal fibroblasts may represent phosphorylated and unphosphorylated forms of the receptor. The effects of phosphorylation of the type I PTH/PTHrP receptor on cell-specific signal transduction and receptor trafficking has not been investigated.
Paracrine epithelial-mesenchymal interactions mediated by PTHrP are increasingly reported in many tissues, but have been most extensively characterized for the developing mouse mammary gland and fetal/neonatal skin (16, 19, 20, 39) Within the mammary gland, the interaction of epithelial-derived PTHrP with type I PTH/PTHrP receptors in stromal cells has been shown to be important for mammary ductal morphogenesis before puberty, lobuloalveolar development during pregnancy and regression of the mammary epithelial bud in males (16, 19, 40). Similarly, expression of PTHrP by alveolar epithelial cells of the mammary gland during late pregnancy may interact with type I PTH/PTHrP receptors in stromal fibroblasts to regulate the differentiation or function of lactating alveolar epithelial cells.

In summary, this study demonstrates the presence of biologically active type I PTH/PTHrP receptors on rat mammary stromal fibroblasts at the end of gestation and suggests a possible paracrine function mediated by PTHrP in regulating the transition to the lactating phenotype.

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Figure 3.1. Morphology of cultured rat mammary stromal fibroblasts by phase-contrast microscopy. Stromal fibroblasts grew as interwoven sheets of spindle-shaped cells. Magnification 290X.
Figure 3.1. Morphology of cultured rat mammary stromal fibroblasts by phase-contrast microscopy.
Figure 3.2. Western blot analysis of protein extracts from cultured rat mammary stromal fibroblasts. Positive controls (+) for vimentin (VIM), smooth muscle actin (SMA), desmin (DES), and pan-cytokeratin (PCK) were tissues homogenates of dermis, stomach, skeletal muscle, and mammary gland, respectively. Rat mammary stromal fibroblasts (FBL) expressed vimentin and smooth muscle actin, but not desmin or pan-cytokeratin. Apparent molecular weights of the immunopositive proteins observed are indicated (kDa).
Figure 3.2. Western blot analysis of protein extracts from cultured rat mammary stromal fibroblasts.
Figure 3.3. Expression of the type I PTH/PTHrP receptor in cultured rat mammary stromal fibroblasts. A) Northern blot analysis of total RNA (20μg) rat kidney (lane 1, positive control) and rat mammary stromal fibroblasts (lane 2). B) Western blot analysis of total cellular proteins extracted from mammary tissue (+, positive control) and rat mammary stromal fibroblasts (FBL) using a polyclonal rabbit anti-PTH receptor antibody (1:100). Apparent molecular weights of proteins immunopositive for the type I PTH/PTHrP receptor are indicated (kDa).
Figure 3.3. Expression of the type I PTH/PTHrP receptor in cultured rat mammary stromal fibroblasts.
Figure 3.4. Adenylyl cyclase stimulation of cultured rat mammary stromal fibroblasts with increasing concentrations of exogenous PTHrP (1-34). Each point represents the mean ± SEM from samples from 1-5 experiments (n=2-15). ( * = P< 0.01 compared to unstimulated cells). (Portions of this assay were completed by Ms. Amy Koh and Dr. Laurie McCauley, Dept. of Periodontics, Prevention, and Geriatrics, University of Michigan, Ann Arbor, MI.)
Figure 3.4. Adenylyl cyclase stimulation of cultured rat mammary stromal fibroblasts with increasing concentrations of exogenous PTHrP (1-34).
Figure 3.5.  Adenylyl cyclase stimulation of cultured rat mammary stromal fibroblasts with increasing concentrations of exogenous bPTH (1-34). Each point represents the mean ± SEM (n=3). (Portions of this assay were completed by Ms. Amy Koh and Dr. Laurie McCauley, Dept. of Periodontics, Prevention, and Geriatrics, University of Michigan, Ann Arbor, MI.)
Figure 3.5. Adenylyl cyclase stimulation of cultured rat mammary stromal fibroblasts with increasing concentrations of exogenous bPTH (1-34).
Figure 3.6. Adenylyl cyclase stimulation of cultured rat mammary stromal fibroblasts. 

bPTH (7-34) (1μM), PTHrP (1-34) (100 nM), or cells stimulated with PTHrP (1-34) (100 nM) after 45 minute pre-incubation with bPTH (7-34) (1 μM). Each point represents the mean ± SEM from 1-5 experiments (n=3-15). (* = P < 0.05 compared to baseline) (Portions of this assay were completed by Ms. Amy Koh and Dr. Laurie McCauley, Dept. of Periodontics, Prevention, and Geriatrics, University of Michigan, Ann Arbor, MI.)
Figure 3.6. Adenylyl cyclase stimulation of cultured rat mammary stromal fibroblasts.
Figure 3.7. Adenylyl cyclase stimulation of cultured cells to PTHrP (1-34) (1 μM) or Forskolin (10 μM). Baseline cAMP levels in unstimulated cells are indicated by the cell name without any additions [rat mammary stromal fibroblasts (RMSF) and ROS 17/2.8 cells (ROS)]. Each point represents the mean ± SEM from 2-5 experiments. (N= 5-15)(* = P < 0.01 compared to baseline, ** = P < 0.001 compared to baseline and RMSF stimulated with PTHrP(1-34)). (Portions of this assay were completed by Ms. Amy Koh and Dr. Laurie McCauley, Dept. of Periodontics, Prevention, and Geriatrics, University of Michigan, Ann Arbor, MI.)
Figure 3.7. Adenylate cyclase stimulation of cultured cells to hPTHrP (1-34) (1 μM) or Forskolin (10 μM) (FSK).
Figure 3.8. Intracellular calcium in cultured rat mammary stromal fibroblasts. The time of addition of agonists is indicated by the arrows; PTHrP (1 μM), bPTH (1 μM), oxytocin (0.2 IU). Traces were completed on the same sample of cells loaded with FURA-2AM (1 μM) and represent the typical trace observed in three or more experiments. (Data were collected and analyzed with the help of Mr. James Hensley, Dr. Charlene Hohl, and Dr. Ruth Altschuld, Department of Medical Biochemistry, and Dr. Susan Mallery, Department of Oral Pathology, Ohio State University, Columbus, OH.)
Figure 3.8. Intracellular calcium in cultured rat mammary stromal fibroblasts.
Figure 3.9. Organization of the rat type I PTH/PTHrP receptor gene. Untranslated regions are shown in white boxes, the signal sequence is designated by the hatched box, exon E1 is designated by a black box, and exons E2 and E3/G are shown as stippled boxes. The positions of the two promoters (P1 and P2) are indicated. Tissues in which the splice variants are observed are indicated. Splice variants associated with decreased surface expression and signal transduction are identified with an asterisk.
Figure 3.9. Organization of the rat type I PTH/PTHrP receptor gene and reported mRNA transcripts
Figure 3.10. Characterization of promoter usage and mRNA splicing of the 5'-region of the type I PTH/PTHrP receptor in cultured rat mammary stromal fibroblasts. A) RT-PCR of 1μg of total RNA isolated from cultured rat mammary stromal fibroblasts (lanes 2-5), rat bone (lanes 6-9), and rat kidney (lanes 10-13). Lane 1: 123 bp DNA ladder. PCR with primers PS and PAS amplify a type I PTH/PTHrP receptor cDNA fragment of 451 bp transcribed from the P2 promoter (P2: lanes 2, 6, 10), PU3 and PAS amplify type I PTH/PTHrP receptor cDNA fragments of 520, 580 and 640 bp transcribed from the P1 promoter in kidney (P1: lanes 3, 7, 11), and P20 and P21 amplify a type I PTH/PTHrP receptor fragment of 167 bp of the coding region (C: lanes 4, 8, 12). RT-PCR with omission of reverse transcriptase was used as a negative control (N: lanes 5, 9, 13). B) Southern blot analysis of RT-PCR products using an α-[32P]dATP-labeled 451 bp gel-purified PCR fragment of the rat type I PTH/PTHrP receptor encoding exons S, E1, E2, and E3.
Figure 3.10. Characterization of promoter usage and mRNA splicing of the 5'-region of the type I PTH/PTHrP receptor transcript in cultured rat mammary stromal fibroblasts.
CHAPTER 4

Regulation of PTHrP mRNA Expression In Cultured Rat Mammary Organoids

Abstract

Parathyroid hormone-related protein (PTHrP) is synthesized by the lactating mammary gland and secreted into the milk in high concentrations; however, little is known about the regulation of PTHrP production during lactation. PTHrP production by the rat mammary gland has been reported to be prolactin responsive in vivo. The purpose of this study was to investigate the regulation of PTHrP mRNA expression by lactogenic hormones (DIP: dexamethasone, insulin, and prolactin) in cultured rat mammary organoids in vitro. Mammary organoids isolated from rats during late pregnancy (d19-21) and lactation (d2-13) were cultured in the presence or absence of DIP. Steady-state levels of PTHrP mRNA were measured and the effect of DIP on the stability of the PTHrP mRNA transcript (141 amino acid isoform) was examined in vitro. No difference was observed in PTHrP mRNA expression between cultured organoids isolated from late pregnant or lactating tissue. Steady state PTHrP mRNA levels in both control (insulin alone) and DIP-treated organoids decreased over the 24-hour culture period. Addition of DIP to the
cultures reduced the subsequent decline in steady state PTHrP mRNA levels compared to controls. Examination of PTHrP mRNA levels in the presence of dexamethasone or prolactin alone indicated that the effects of DIP on PTHrP mRNA were mediated primarily by dexamethasone. Administration of either 5,6-dichloro-benzimidazole riboside (DRB) or cycloheximide, in the presence or absence of DIP, increased steady-state PTHrP mRNA levels relative to controls throughout the 24-hr culture period. Cytoplasmic proteins isolated from DIP-treated cultures did not increase the half-life of PTHrP mRNA in vitro. The data revealed that stabilization of the PTHrP mRNA transcript is an important mechanism involved in PTHrP production by mammary tissue, and suggested that 1) the synthesis of a labile protein is required for degradation of the PTHrP mRNA transcript, and 2) the effects of DIP on PTHrP mRNA are mediated, in part, by a post-transcriptional mechanism.

Introduction:

Since the discovery of parathyroid hormone-related protein (PTHrP) as a principal factor responsible for humoral hypercalcemia of malignancy, PTHrP production has been demonstrated in many normal tissues (1). The highest levels of PTHrP have been reported in the lactating mammary gland where milk concentrations of PTHrP are 1000 to 10,000-fold greater than in plasma (2, 3). Despite the high level of PTHrP production, the regulation of PTHrP expression in mammary tissue during lactation is poorly understood.
The effects on PTHrP production by various growth factors and cytokines known to be important regulators of mammary morphogenesis, growth, and differentiation have been investigated. PTHrP production by the rat mammary gland has been reported to be increased by prolactin in vivo (4-6). Production of PTHrP was induced by injection of prolactin or by suckling in lactating rats, and PTHrP mRNA levels declined rapidly after removal of the suckling stimulus (4-6). In cultured mammary cells from lactating cows, prolactin induced PTHrP production; however, prolactin had no effect on PTHrP production in human mammary cells in vitro (7, 8). In addition, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) have been reported to increase PTHrP production in cultured mammary cells (8-10). Insulin increased the transcription of PTHrP mRNA in cultured human mammary epithelial cells (8). In human keratinocytes, EGF has been reported to increase both PTHrP mRNA transcription and stability (11). The effects of prolactin on PTHrP mRNA expression in mammary tissue have not been investigated in vitro.

The purpose of this study was to investigate the regulation of PTHrP mRNA expression by lactogenic hormones in cultured rat mammary organoids to determine if the effects of these hormones are mediated at the level of messenger RNA stability.

Materials and Methods

Isolation of rat mammary organoids: Rat mammary organoids were obtained from mammary tissue of late pregnant (d19-21) and lactating (d2-13) Sprague-Dawley rats.
Briefly, mammary tissue was aseptically harvested and minced in chilled serum-free Dulbecco's modified Eagle:F12 medium (Gibco-BRL; Gaithersburg, MD) with 50 μg/ml gentamicin sulfate. Minced tissue was incubated for 1.5 hours on a rotary shaker (150 rpm) at 37°C in SFM containing 2 mg/ml of collagenase (Type III, Worthington Biochemical Corporation, Lakewood, NJ.) The tissue suspension was centrifuged at 100 xg to pellet organoids and larger cell clumps. The pellet was resuspended in 30 ml of serum-containing medium [DMEM:F12 with 10% FBS and gentamicin sulfate (50 μg/ml)], washed, and filtered through a 40 micron pore nylon mesh filter (Tetko, Inc., Briarcliff Manor, NY). Retained organoids were collected and aliquoted to 6-well tissue culture plates (100 μl of packed cells/well). Organoids were incubated in medium [DMEM:F12 containing 10% FBS and insulin (5 μg/ml)] for 2 hours in 5% CO₂ at 37°C. Medium was then changed to low-serum conditions [DMEM:F12 with 2% FBS, insulin (5 μg/ml), and gentamicin sulfate (50 μg/ml)] before dexamethasone (5 μg/ml), ovine prolactin (2 μg/ml), cycloheximide (10 μg/ml), or 5,6-dichloro-benzimidazole riboside (DRB) (100μM) were added.

**Northern Blot Analysis:** Total RNA was extracted from the cultured mammary organoids using Trizol (Gibco-BRL), quantitated, and stored at -80°C. Total RNA (20 μg) was separated by gel electrophoresis in a 1.2% agarose/2.2 M formaldehyde gel and transferred overnight to a nylon membrane (Duralon UV, Stratagene, La Jolla, CA) in 20X SSC (3M
sodium chloride and 0.3M sodium citrate). The membrane was incubated in prehybridization solution [50% formamide, 5X SSC, 10 mM Tris-HCl (pH 7.5), 125 µg/ml salmon sperm DNA, 1X Denhardt’s solution, 0.2% SDS and 1% background quencher (Molecular Research Center, Inc., Cincinnati, OH)] for 2 hours at 42°C. [32P]-dATP-labeled 434-bp bovine PTHrP cDNA probe was added and hybridization was continued overnight at 42°C. The membrane was washed in 1X SSC and 0.1% SDS at 50°C for 1 hour. Blots were stripped by incubating in 200mM Tris-Cl pH 7.0, 0.1X SSC, 0.1% SDS for 1 hour at 60°C and reprobed with an [32P]-dATP-labeled 225 bp rat GAPDH cDNA probe. Radioactivity was measured using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). PTHrP mRNA was normalized to GAPDH mRNA in each sample and plotted using Graph Pad Prism.

**Cytoplasmic Protein Isolation:** Proteins were isolated from mammary organoids after 2 hr treatment with insulin alone (control) or DIP [dexamethasone (5µg/ml), insulin (5µg/ml) and prolactin (2µg/ml)]. Organoids were harvested and resuspended in 2-3 packed cell volumes of homogenization buffer [10 mM Tris (pH 7.4), 0.5 mM DTT, 10 mM KCl, 1.5 mM MgCl₂]. After incubation on ice for 10 min, samples were homogenized, and the homogenate was centrifuged at 14,000 xg to pellet out the nuclei. The supernatant was transferred to a new tube and 0.11 volume of 10X extraction buffer was added [100 mM Tris (pH 7.4), 50 mM DTT, 1.5 M KCl, 15 mM MgCl₂]. Samples were centrifuged at 100,000 xg in a Beckmann L5-65B ultracentrifuge to pellet the ribosomal fraction. The
supernatant containing cytoplasmic proteins was aliquoted, snap frozen in liquid N₂, and stored at -80°C. Proteins were quantitated using the Bradford assay (13).

**Synthesis of PTHrP mRNA transcript:** Linearized PTHrP mRNA transcript was prepared by Shelly Kendrick Ph.D., Dept. of Veterinary Biosciences, Ohio State University, Columbus, OH. A cDNA clone of the 141 amino acid isoform of human PTHrP mRNA was obtained from Dr. Philbrick (Dept. Of Internal Medicine, Yale University Medical School, New Haven, CT). The full length PTHrP clone including a 40 nt poly(A)* tail was subcloned into the SacI site of pGEM-3Z (Promega, Madison, WI). Plasmid was linearized by digesting with PstI and KpnI, extracted with phenol:chloroform, precipitated with 100% ethanol, air-dried, dissolved in sterile water and quantitated. Radioactive PTHrP mRNA probe was synthesized *in vitro* using the Ambion Maxiscript kit (Ambion Inc., Austin, TX). The linearized PTHrP cDNA template (1 µg) was transcribed for 1 hr at 37°C using T7 RNA polymerase (10U), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 2 mM of m⁷G(5')ppp(5')G cap analog (Ambion), 0.2 mM UTP, and 50 µCi of [³²P]-UTP in transcription buffer. RNase-free DNase (1 µl) was added and the incubation was continued at 37°C for 15 min. The mRNA probe was precipitated at -20°C for 30 min after the addition of 20 µg tRNA, 125 mM ammonium acetate, and 3 volumes of 100% ethanol. The mRNA probe was centrifuged at 12,000 xg for 30 min, washed in 70% ethanol, air-dried, and resuspended in 50 µl of DEPC-treated water.
**In Vitro mRNA Stability Assay:** Cytoplasmic proteins (50 μg) from control and DIP treated cultures were incubated with [³²P]-UTP- labeled PTHrP mRNA transcript (2 X 10⁶ cpm) in homogenization buffer (final volume 150 μl) at 30°C. Aliquots of the reaction (6 μl) were removed at selected time points and placed in stop solution (32 μl) (500 mM ammonium acetate, 100 mM EDTA, pH 8). Triplicate samples were extracted with an equal volume of phenol:chloroform, precipitated with isopropanol (40 μl) and stored at -20°C. After the last time point was collected, each set of replicate samples was washed in 70% ethanol, air-dried, dissolved in DEPC-treated water (10 μl) and evaluated by northern blot analysis. Radioactivity was measured using a PhosphorImager (Molecular Dynamics, Inc.) The half-life of the mRNA transcript was determined by linear regression analysis on semilog plots using GraphPad Prism. Results were analyzed for statistical significance by a two-tailed Student’s t-test using GraphPad Instat.

**Results**

**Effects of Lactogenic Hormones on Steady-State PTHrP mRNA in Cultured Mammary Organoids:** No differences in steady-state PTHrP mRNA levels were observed between organoids isolated from late pregnant and lactating rat mammary glands. Therefore, data from both types of cultures was combined in these analyses.

Steady-state PTHrP mRNA levels decreased over the 24-hour culture period in both DIP-treated (dexamethasone, insulin, and prolactin) and control (insulin alone) organoid cultures, however, the changes were not statistically significant (Fig. 4.1). The addition of
DIP to the cultures increased steady-state PTHrP mRNA levels 3-fold throughout the culture period relative to the controls, suggesting that DIP administration caused an increase in PTHrP mRNA transcription or stability. The effects of dexamethasone and prolactin on steady-state PTHrP mRNA levels were evaluated independently (Fig. 4.2). Addition of dexamethasone or prolactin alone resulted in increased steady-state PTHrP mRNA levels compared to the control. The effect of DIP on PTHrP mRNA levels was intermediate between the effects of dexamethasone or prolactin alone, suggesting that the effect of DIP is mediated primarily by dexamethasone.

To determine if the effect of DIP on steady-state PTHrP mRNA levels resulted from increased mRNA stability, 5,6-dichloro-benzimidazole riboside (DRB), which inhibits elongation of the transcription complex, was added. Administration of DRB to cultures alone or in the presence of DIP resulted in stabilization of the steady-state PTHrP mRNA levels throughout the 24-hr culture period (Fig. 4.3). At 24 hours, PTHrP mRNA levels compared to T₀ were 82%, 40%, 27%, and 8% for DIP and DRB, DRB alone, DIP alone, and control cultures, respectively. The effect of DRB on steady-state PTHrP mRNA suggests that the synthesis of a labile protein is required for degradation of the PTHrP mRNA transcript in the cultured mammary organoids. Combined treatment with DIP and DRB resulted in higher steady-state PTHrP mRNA levels than treatment with DIP or DRB alone, suggesting that DIP stabilizes PTHrP mRNA by a post-transcriptional mechanism.

The administration of cycloheximide (CHX) alone or in the presence of DIP resulted in a similar increase of steady-state PTHrP mRNA levels throughout the culture
period relative to controls (Fig. 4.4). At 24 hours, PTHrP mRNA levels compared to $T_0$ were 88%, 59%, 27, and 8% for DIP and CHX, CHX alone, DIP alone, and control cultures, respectively. The effect of cycloheximide alone on steady-state PTHrP mRNA levels was more pronounced than the effect of DIP, and suggests that either protein synthesis is required for PTHrP mRNA degradation or inhibition of PTHrP mRNA transcription. Combined treatment with DIP and cycloheximide had a greater effect on PTHrP mRNA levels than treatment with either DIP or cycloheximide alone, suggesting that the effects of DIP and cycloheximide on steady-state PTHrP mRNA are mediated by different mechanisms.

Effects of Lactogenic Hormones on PTHrP mRNA Stability In Vitro: No differences in PTHrP mRNA stability were observed between organoids isolated from late pregnant and lactating rat mammary glands. Therefore, data from both types of cultures was combined in these analyses.

Cytoplasmic proteins isolated from DIP-treated organoid cultures did not stabilize the PTHrP mRNA transcript in vitro, compared with the control (Fig. 4.5). A biphasic mRNA decay mechanism was apparent in both control and DIP-treated cultures (Fig. 4.6). The half-lives for the initial, rapid phase of mRNA decay were 70 and 65 min in DIP-treated and control cultures, respectively. Similarly, the half-lives for the second slow phase of mRNA decay were 4.5 and 6 hours for the DIP-treated and control cultures, respectively.
Discussion

This study demonstrated that stabilization of the PTHrP mRNA transcript is an important mechanism for increasing PTHrP mRNA levels within the cell. Addition of cycloheximide or DRB to the mammary organoids isolated from late pregnant and lactating rat mammary glands resulted in increased steady-state levels of the PTHrP mRNA over the 24-hr culture period relative to controls, suggesting that the synthesis of a protein(s) is required for destabilization of the PTHrP transcript in mammary tissue. An additive effect on PTHrP mRNA levels was observed with the addition of lactogenic hormones (DIP), and these effects were mediated primarily by dexamethasone. The specific mechanism by which DIP or dexamethasone treatment regulated steady-state levels of the PTHrP mRNA transcript was not determined.

A 3 to 25-fold induction of PTHrP mRNA transcription in response to prolactin administration or suckling has been reported in lactating rats (4, 6). In this study, a prominent increase in steady-state PTHrP mRNA levels in cultured rat mammary organoids was not observed in response to either insulin, dexamethasone, or prolactin alone or in combination (DIP). Although steady-state mRNA levels do not directly measure transcription, our results suggest that these agents do not significantly increase PTHrP mRNA transcription. The mammary organoids used in this study should retain the overall three-dimensional cell-to-cell orientation observed in vivo; however, attachments to basement membranes or substratum have been disrupted. The expression of mammary proteins has been shown to be regulated by the interaction of cells with extracellular matrix
components. Furthermore, the normal three-dimensional tissue architecture has been demonstrated to be important for these interactions (14-16). It may be that induction of PTHrP mRNA transcription in mammary tissue requires a similar interaction with the extracellular matrix.

Regulation of PTHrP mRNA transcription and stability by cycloheximide has been reported previously (16,17). These effects of cycloheximide on mRNA transcription and stability are commonly observed in members of the early response gene family, such as c-fos (17). PTHrP mRNA shares many features of early response genes including multiple copies of AU-rich instability sequences present within the 3'-untranslated region of the message. Cycloheximide slowed the decline of steady-state levels of PTHrP mRNA, but its specific effects on the induction of PTHrP mRNA transcription were not determined in this study. The decreased synthesis of a repressor protein is the proposed mechanism of induction of gene transcription by cycloheximide (16). Three mechanisms have been proposed for the increased mRNA stability of early response genes observed with cycloheximide: 1) the decreased synthesis of a labile ribonuclease, 2) decreased co-translational degradation, 3) decreased synthesis of a kinase or phosphatases which regulates the phosphorylation of a protein involved in mRNA stabilization (16, 18, 19). Our data is consistent with the first and third mechanisms of mRNA stabilization; and suggests that these effects may be mediated at the level of mRNA transcription. The degradation of the PTHrP mRNA transcript in the in vitro mRNA stability assay, indicates
that co-translational destabilization of the PTHrP mRNA transcript is not required for its degradations.

DIP treatment regulated the expression of PTHrP mRNA in the mammary organoids by a transcriptional or post-transcriptional mechanism. Because of the lack of a marked induction of PTHrP mRNA transcription by DIP and the effects of DRB alone on PTHrP mRNA levels, the effects of DIP on PTHrP mRNA stability in organoid cultures could not be evaluated. However, since the combined effects of DIP and DRB on steady-state PTHrP mRNA levels were greater than those observed for DIP or DRB alone, DIP appeared to stabilize PTHrP mRNA by a post-transcriptional mechanism. In contrast, the lack of an effect of DIP treatment on PTHrP mRNA stability in the in vitro assay suggests that the effect of DIP on steady-state mRNA levels observed in culture is mediated at the level of mRNA transcription although at a low level, and that DIP induction of the synthesis of proteins required for stabilization of the PTHrP mRNA transcript did not occur. However, the effects of cellular processes involved in mRNA transport or localization on PTHrP mRNA stability could not be evaluated in the in vitro assay (19-21). Similarly, post-translational regulation of proteins involved in mRNA stabilization or degradation may be different within the cell than in the in vitro assay (19, 22-24).

In summary, this study demonstrated that stabilization of the PTHrP mRNA transcript may be an important mechanism for regulating PTHrP mRNA production in mammary tissue. Furthermore, destabilization of the PTHrP transcript appears to be mediated by a labile protein. The effects of lactogenic hormones in this system were
mediated primarily by dexamethasone, by a transcriptional or post-transcriptional mechanism.

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Figure 4.1. Effects of DIP [dexamethasone (5 μg/ml), insulin (5 μg/ml), and ovine prolactin (2 μg/ml)] on steady-state PTHrP mRNA levels in cultured mammary organoids. DIP was administered at time zero. PTHrP mRNA levels were normalized to GAPDH mRNA levels before calculating the percentage relative to time zero. Cultures in medium with insulin alone served as the control (CON).
Figure 4.1. Effects of DIP [dexamethasone (5 μg/ml), insulin (5 μg/ml), and ovine prolactin (2 μg/ml)] on steady-state PTHrP mRNA levels in cultured mammary organoids.
Figure 4.2. Effects of DIP [dexamethasone (5 μg/ml), insulin (5 μg/ml), and ovine prolactin (2 μg/ml)], dexamethasone (DEX) (5 μg/ml), and ovine prolactin (PRL) (2 μg/ml) on steady-state PTHrP mRNA levels in cultured mammary organoids. Treatments were administered at time zero. PTHrP mRNA levels were normalized to GAPDH mRNA levels before calculating the percentage relative to time zero. Cultures in medium with insulin alone served as the control (CON).
Figure 4.2. Effects of DIP [dexamethasone (5 μg/ml), insulin (5 μg/ml), and ovine prolactin (2 μg/ml)], dexamethasone (DEX) (5 μg/ml), and ovine prolactin (PRL) (2 μg/ml) on steady-state PTHrP mRNA levels in cultured mammary organoids.
Figure 4.3. Effects of inhibition of transcription with 5,6-dichloro-benzimidazole riboside (DRB) on steady-state PTHrP mRNA levels in cultured mammary organoids. DRB (100 μM) in the presence or absence of DIP [dexamethasone (5 μg/ml), insulin (5 μg/ml), and ovine prolactin (2 μg/ml)] was administered at time zero. PTHrP mRNA levels were normalized to GAPDH mRNA levels before calculating the percentage relative to time zero. Cultures in medium with insulin alone served as the control (CON).
Figure 4.3. Effects of inhibition of transcription with 5,6-dichloro-benzimidazole riboside (DRB) on steady-state PTHrP mRNA levels in cultured mammary organoids.
Figure 4.4. Effects of inhibition of protein synthesis by cycloheximide (CHX) on steady-state PTHrP mRNA levels in cultured mammary organoids. Cycloheximide (10 μg/ml) in the presence or absence of DIP [dexamethasone (5 μg/ml), insulin (5 μg/ml), and ovine prolactin (2 μg/ml)] was administered at time zero. PTHrP mRNA levels were normalized to GAPDH mRNA levels before calculating the percentage relative to time zero. Cultures in medium with insulin alone served as the control (CON).
Figure 4.4. Effects of inhibition of protein synthesis by cycloheximide (CHX) on steady-state PTHrP mRNA levels in cultured mammary organoids.
Figure 4.5. Effects of DIP treatment on PTHrP mRNA stability in vitro. Cytoplasmic proteins were isolated from DIP-treated cultures [dexamethasone (5 μg/ml), insulin (5 μg/ml), and ovine prolactin (2 μg/ml)] after 2 hr of incubation. Protein extracts (50 μg) were incubated with [³²P]-UTP-labeled PTHrP mRNA transcript (2 × 10⁶ cpm). Triplicate samples were removed at various time points and analyzed for the presence of PTHrP mRNA transcript. PTHrP mRNA levels were calculated as a percentage of the levels at time zero. Protein extracts isolated from cultures treated with insulin alone were used as the control (CON).
Figure 4.5. Effects of DIP treatment on PTHrP mRNA stability in vitro.
Figure 4.6. Determination of the half-life of PTHrP mRNA in vitro. Half-lives were calculated by linear regression analysis of the data presented in figure 5 plotted on a semilogarithmic scale. DIP [dexamethasone (5 μg/ml), insulin (5 μg/ml), and ovine prolactin (2 μg/ml)]. CON [insulin (5 μg/ml)].
Figure 4.6. Determination of the half-life of PTHrP mRNA \textit{in vitro}.

\( T_{1/2} = 70 \text{ min} \)

\( T_{1/2} = 4.5 \text{ hr} \)

\( T_{1/2} = 65 \text{ min} \)

\( T_{1/2} = 6 \text{ hr} \)


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