Destabilization of parathyroid hormone mRNA by extracellular Ca^{2+} and the calcimimetic R-568 in parathyroid cells: role of cytosolic Ca and requirement for gene transcription

Cynthia S Ritter, Sangeeta Pande, Irina Krits, Eduardo Slatopolsky and Alex J Brown

Renal Division, Washington University Medical School, Box 8126, 660 S. Euclid, St Louis, Missouri 63110, USA

(Correspondence should be addressed to A J Brown; Email: brownlab@im.wustl.edu)

Abstract

Extracellular Ca reduces parathyroid hormone (PTH) levels through several mechanisms, but many details of the intracellular steps involved have been difficult to elucidate because of the lack of a suitable parathyroid cell model. The present studies utilized our Ca-responsive bovine parathyroid organoid culture system (pseudoglands) to examine PTH mRNA in intact parathyroid cells. Increasing medium calcium from 0.4 to 3.0 mM reduced PTH mRNA to 20–30% of basal by 16 h. Reducing medium Ca from 3.0 to 0.4 mM restored PTH mRNA levels over a 24-h period. PTH mRNA was also reduced by the calcimimetic R-568, confirming the role of the calcium-sensing receptor. PTH decay rates were determined by placing pseudoglands in either 0.4 or 3.0 mM Ca for 2 h and then blocking gene transcription. PTH mRNA remained stable for at least 24 h in pseudoglands incubated in 0.4 mM Ca, but fell gradually by 62% in the presence of 3.0 mM Ca. Blocking transcription prior to the addition of high-Ca medium dramatically blunted the Ca-induced degradation of PTH mRNA, indicating that acceleration of PTH mRNA decay by Ca requires gene transcription. Pharmacologic investigation of the signaling pathways involved indicated that the Ca-induced reduction of PTH mRNA did not involve MAP kinase, phospholipase D, or cyclic AMP. However, increasing cytosolic Ca with thapsigargin or the Ca ionophore A23187 decreased PTH mRNA levels. In summary, Ca-mediated destabilization of PTH mRNA requires gene transcription and involves increases in cytosolic Ca.

Journal of Molecular Endocrinology (2008) **40**, 13–21

Introduction

The parathyroid glands play a central role in Ca homeostasis by secreting the appropriate amount of the calciotropic hormone parathyroid hormone (PTH) to maintain or correct serum Ca levels (Brown *et al.* 1993, Chattopadhyay *et al.* 1996, Chen & Goodman 2004). Chief cells of the parathyroid gland express a cell surface calcium-sensing receptor (CaR) that detects small changes in serum calcium and controls the rate of PTH secretion (Fukugawa & Kurokawa 2002, Hofer & Brown 2003). Regulation of PTH secretion by Ca is complex and involves several mechanisms that allow the parathyroid glands to adapt both acutely and chronically to the need for Ca. Studies in freshly dispersed parathyroid cells have demonstrated that high extracellular Ca suppresses the release of pre-packaged PTH and stimulates PTH peptide degradation (Habener *et al.* 1975). *In vivo* studies have shown that dietary Ca can regulate PTH mRNA stability and parathyroid gland growth, but examination of the mechanisms involved in long-term control of parathyroid cell function by calcium has been hampered by the lack of an appropriate *in vitro* model; primary monolayer cultures of bovine parathyroid cells lose their response to calcium (Brown *et al.* 1995, Mithal *et al.* 1995) within days of being placed in culture, and no parathyroid cell

lines that secrete PTH in a normally regulated fashion have been established.

Recent studies using *ex vivo* parathyroid extracts and exogenous cell lines have broadened our understanding of the mechanism of PTH mRNA decay by Ca. Using *in vitro* degradation assays, Moallem *et al.* (1998) showed that an increase in PTH mRNA levels in hypocalcemic rats was due to post-transcriptional stabilization of PTH mRNA involving changes in the protein–RNA interactions that affect PTH mRNA degradation. Parathyroid cytosolic proteins from hypocalcemic rats bind to a discrete element in the rat PTH 3′-untranslated region (UTR) that serves to stabilize the PTH transcript. While these studies have given valuable insight into PTH mRNA regulation, demonstration of a long-term, direct effect of Ca on PTH mRNA stability has not been demonstrated in intact parathyroid cells.

We have recently developed a three-dimensional culture system in which bovine parathyroid cells dispersed in collagen coalesce into small organoids, termed pseudoglands, which retain a stable response to Ca for several weeks (Ritter *et al.* 2004). Ca acutely suppressed PTH secretion with a half maximal inhibition (i.e., set point) of 1.05 mM, similar to that observed *in vivo* and in freshly dispersed cells, making this model ideal for studying the direct effect of Ca on PTH transcript stability and for investigating the

intracellular signaling pathways activated by the CaR. In the present study, the bovine pseudogland model was used to examine Ca-dependent control of PTH mRNA stability for the first time in intact parathyroid cells. In addition, the endogenous signaling pathways that mediate this regulation were investigated.

Materials and methods

Bovine parathyroid pseudogland culture

Bovine parathyroid glands were obtained from MBH Enterprises (Tampa, FL, USA) and cultured in type I collagen as described previously (Ritter *et al.* 2004). Briefly, bovine parathyroid glands were sliced to 0.5 mm thickness with a tissue slicer (Stadie Riggs, Thomas Scientific, Swedesboro, NJ, USA) and placed in a 1:1 mixture of Dulbecco's modified Eagle's medium (DME):Ham's F-12 medium containing 0.5 mM Ca, DNase I (10 µg/ml), and collagenase (2300 U/ml; collagenase XI-S, Sigma-Aldrich). The suspension (10 ml/g tissue) was agitated in a shaking water bath at 37 °C for 90 min with periodic pipeting to disaggregate the tissue. The digested material was washed three times with serum-free medium DME:Ham's F-12 (1:1, 1 mM Ca), 15 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml holo-transferrin, 2 mM glutamine, 1% nonessential amino acids, and 0.1% BSA). This medium was used for routine culturing, with alterations in the concentration of Ca as specified. To prepare the pseudoglands, the dispersed cells were placed on ice and mixed with a 1:1 mixture of 2× Krebs-Henseleit buffer and rat-tail type I collagen (BD Biosciences, Franklin Lakes, NJ, USA; final collagen concentration of 1.45 mg/ml), and then placed in 24-well culture plates (200 000 cells in a volume of 0.25 ml/well). The collagen was allowed to solidify at 37 °C in a 5% CO₂ incubator, and then 0.25 ml of serum-free medium was carefully layered on top. The cultures were left undisturbed until cell coalescence was complete (~2 weeks).

Investigation of signaling pathways

Calcium affects a variety of signaling pathways in parathyroid cells. Inhibitors and activators of these pathways were used to investigate their importance in the bovine parathyroid pseudogland cultures. All test reagents were reconstituted as per the manufacturer instructions and added to the cultures at the specified concentrations. The concentration (or range of concentrations) for each test reagent was based on general usage in primary cultures or cell lines, as obtained from recently published literature. Unless stated otherwise, the reagents were examined in both

low- and high-Ca conditions. Reagents were analyzed in an average of two separate experiments (1–5 experiments each; data from representative experiments are shown in the figures). Treatment with vehicle (dimethyl sulfoxide (DMSO) or EtOH, where appropriate) served as control. Unless stated otherwise, the parathyroid pseudogland cultures were placed in 0.4 mM Ca for 24 h to maximize PTH mRNA levels. Test reagents were then added 30 min prior to challenge with 3.0 mM Ca; PTH mRNA was measured after 24 h. The bovine parathyroid pseudogland cultures were treated with the following reagents: R-568 and S-568 (Amgen); pertussis toxin (Sigma-Aldrich); dibutyl cAMP (Sigma-Aldrich); PD 98059 (Calbiochem, San Diego, CA, USA), and SB 203580 (Sigma-Aldrich); Y-27632 (Calbiochem); *n*-butanol (Fischer Scientific, Pittsburgh, PA, USA); thapsigargin (Sigma-Aldrich); and A23187 (Calbiochem).

PTH mRNA analysis

Pseudoglands treated as specified in Results were homogenized in RNazol Bee (Tel-Test, Friendswood, TX, USA) and total RNA was isolated as directed by the manufacturer. Initial studies utilized a ribonuclease protection assay to quantify PTH mRNA and 28S rRNA. The bovine PTH riboprobe was transcribed from a template prepared by RT-PCR of the coding region of bovine parathyroid cDNA using T7 RNA polymerase and ³²P-CTP as described previously (Brown *et al.* 1995, Ritter *et al.* 2004). The human 28S rRNA probe was transcribed using T7 RNA polymerase, ³²P-CTP, and a template purchased from Ambion. The ribonuclease protection assay was performed as detailed previously (Brown *et al.* 1995, Ritter *et al.* 2004). Briefly, RNA was mixed with the ³²P-labeled riboprobes (500 000 c.p.m.) for PTH mRNA and 28S rRNA (Ambion Inc., Austin, TX, USA), and hybridized at 45 °C for 16 h. The samples were digested with ribonuclease T1 and then with proteinase K. The protected fragments were extracted with phenol-chloroform, precipitated with ethanol, and resolved on a 5% TBE-urea gel (Bio-Rad, Hercules, CA, USA). The gel was dried and the bands were visualized and quantified using a phosphorimager (Molecular Dynamics/GE Healthcare, Piscataway, NJ, USA; model 445 SI). Data are expressed as the ratio of PTH mRNA/28S RNA to normalize for variations in loading. Unless stated otherwise, triplicate samples (with three to four pseudoglands combined per sample) were analyzed for each data point.

Subsequent studies that investigated signaling pathways mediating the effects of Ca on PTH mRNA were performed using northern blot analysis. RNA was resolved on 1.2% agarose/formaldehyde gel and transferred to nylon membrane (Zeta-Probe, Bio-Rad) by capillary action. The membranes were u.v.-cross-

linked (Stratalinker, Stratagene, La Jolla, CA, USA) and pre-hybridized in 7% SDS, 50% formamide, 0.15 M NaCl, 0.12 M sodium phosphate (pH 7.0), and 1 mM EDTA at 60 °C for 30 min. 32 P-labeled PTH riboprobes, prepared as above, was added at a 10^6 c.p.m./ml, and the hybridization was continued overnight. The membranes were washed three times with $0.1 \times$ SSC/0.1% SDS for 30 min at 60 °C, and then exposed to a phosphorimager screen overnight and band intensities were quantified. The membranes were reprobbed with 32 P-labeled riboprobes for 18S rRNA or 28S rRNA using templates from Ambion. Hybridization and washing were performed as for PTH mRNA. The data are expressed as the ratio of PTH mRNA/18S or PTH mRNA/28S rRNA to normalize for variations in loading and transferring. Triplicate samples (two pseudoglands combined per sample) were analyzed for each data point. Data were analyzed using GraphPad Instat software (GraphPad, San Diego, CA, USA), utilizing ANOVA and Tukey–Kramer multiple

comparisons test *post hoc* analysis, or unpaired *t*-test programs, where applicable.

Results

Time courses for regulation of PTH mRNA by Ca

We previously observed a fivefold regulation of PTH mRNA by Ca after a 72-h incubation of pseudoglands in 0.4 vs 3.0 mM Ca (Ritter *et al.* 2004). In the present study, a time course was carried out to determine the time required for the Ca regulation of PTH mRNA. Bovine pseudoglands were placed in medium containing 3.0 mM Ca and RNA analyzed for PTH mRNA and 28S rRNA after 0, 2.5, 8, 16, and 40 h. As shown in Fig. 1, following a lag of several hours, PTH mRNA decreased to a nadir at about 16 h. The reversibility of the Ca suppression of PTH mRNA was assessed by switching from 3.0 to 0.4 mM Ca after 16 h and then analyzing for PTH mRNA after 2.5, 8, and 24 h. Reversal

Figure 1 Time course for changes in PTH mRNA in response to extracellular Ca. Bovine parathyroid pseudoglands maintained in serum-free medium containing 1.0 mM Ca were switched to medium containing 3.0 mM Ca for 0, 2.5, 8, 16, and 40 h. After 16 h of 3.0 mM Ca, a portion of the pseudoglands were placed in 0.4 mM Ca for 2.5, 8, and 24 h. PTH mRNA and 28S rRNA were determined by ribonuclease protection assay and the quantified bands expressed as a ratio. Data are given as mean $\pm$ s.d. ($n=3$ samples per time point; ANOVA, Tukey–Kramer post-analysis). * $P \leq 0.05$ vs 0 h in 3 mM Ca; ** $P \leq 0.05$ vs 16 h in 3 mM Ca.

of the effects of Ca was evident within 2.5 h, with a gradual increase over a 24-h period.

Ca accelerates PTH mRNA decay

The effect of extracellular Ca on PTH mRNA stability was assessed using an inhibitor of RNA transcription, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB; Sigma). DRB inhibits RNA polymerase II, causing premature termination of transcription. Bovine parathyroid pseudoglands were pre-incubated in medium containing either 0.5 or 3.0 mM Ca for 2 h. DRB (20 μ g/ml) was then added, and the pseudoglands analyzed for PTH mRNA and 28S rRNA after 0, 4, 8, and 24 h. As shown in Fig. 2A, PTH mRNA was completely stable in pseudoglands incubated in medium containing 0.4 mM Ca, but was degraded with a half-life of $\sim$ 12 h in pseudoglands incubated in medium containing 3.0 mM Ca. The significant decay of PTH mRNA seen with high Ca suggests that during the 2-hr pre-incubation, a factor is induced that is critical for this decay.

Ca regulation of PTH mRNA stability requires gene transcription

To determine whether the factor that is critical for Ca-mediated degradation of PTH mRNA is induced transcriptionally by Ca, we pre-incubated the pseudoglands in medium containing 1 mM Ca with or without DRB for 30 min. The pseudoglands were then placed in medium containing 3 mM Ca with or without DRB for 16 h. As shown in Fig. 2B, the pseudoglands placed in 3 mM Ca with DRB showed little degradation of the PTH mRNA over 16 h. However, the pseudoglands placed in medium without DRB showed a 56% decrease in PTH mRNA, confirming that gene transcription is necessary for Ca-mediated degradation of PTH mRNA. Similar results were obtained using actinomycin D, another commonly used RNA transcription inhibitor that inhibits transcription by binding DNA at the transcription initiation complex, thus preventing elongation by RNA polymerase (data not shown).

Investigation of signaling pathways

To confirm the role of the CaR in the regulation of PTH mRNA stability by extracellular Ca, pseudoglands were treated with 3.0 or 0.4 mM Ca containing the calcimimetic R-568 or its less active isomer S-568 (which served as a control) for 20 h. As shown in Fig. 3, R-568 significantly reduced PTH mRNA by 60%; 3.0 mM Ca significantly reduced PTH mRNA by 76%. The less active isomer had no significant effect on PTH mRNA. These findings show, for the first time, a direct effect of the active calcimimetic R-568 on reduction of PTH mRNA in intact parathyroid

Figure 2 (A) Ca accelerates PTH mRNA decay in parathyroid cells. Bovine parathyroid pseudoglands were placed in media containing 0.4 or 3.0 mM Ca. After 2 h, an inhibitor of RNA polymerase II, D-ribofuranosylbenzimidazole (DRB, 20 μ g/ml), was added and PTH mRNA and 28S rRNA were measured by ribonuclease protection assay after 0, 4, 8, and 24 h. Data are given as mean $\pm$ s.d. ($n=4$ samples per time point; ANOVA, Tukey–Kramer post-analysis). * $P<0.05$, ** $P<0.001$ versus time 0. (B) Reduction of PTH mRNA by calcium requires gene transcription. Bovine parathyroid pseudoglands maintained in medium containing 1.0 mM Ca were pre-incubated in DMSO vehicle or DRB (20 μ g/ml) for 30 min; medium was then replaced with 3 mM Ca with vehicle or DRB for 16 h. The transcription inhibitor DRB blocked the Ca-mediated decay of PTH mRNA, confirming that gene transcription is necessary for Ca to accelerate the degradation of PTH mRNA. PTH mRNA and 28S rRNA were measured by ribonuclease protection assay. Data are given as mean $\pm$ s.d. ($n=3$ samples per time point; unpaired t -test analysis); * $P<0.01$ versus + DRB.

cells. To examine the effect of the active calcimimetic on PTH mRNA stability, bovine parathyroid pseudoglands were incubated with 0.4 mM Ca with or without R-568 for 2 h, and then exposed to the transcriptional inhibitor actinomycin D (1 μ g/ml) for 20 h. As shown in Fig. 4A, R-568 significantly increased the PTH mRNA decay rate compared with the 0.4 mM Ca control. Therefore, the reduction in PTH mRNA by R-568 can be attributed, in part, to acceleration of transcript degradation. In addition, we found that inhibiting gene transcription with actinomycin D prior to treatment with R-568 partially blocked the reduction of PTH mRNA by the calcimimetic (Fig. 4B), indicating that maximal reduction of PTH mRNA by R-568 requires gene transcription.

Figure 3 Calcimimetic R-568 decreases PTH mRNA levels. Bovine parathyroid pseudoglands were maintained in serum-free medium containing 0.4 mM Ca for 24 h to maximize basal secretion of PTH, and then treated for 20 h with 0.4 mM, 3.0 mM, 0.4 mM Ca with R-568 (10 nM), or 0.4 mM Ca with S-568 (inactive stereoisomer, 10 nM). DMSO was used as vehicle. PTH mRNA and 18S rRNA were measured by northern blot analysis. Data are given as mean $\pm$ s.d. ($n=3$ samples per condition; unpaired t -test analysis); * $P<0.001$ vs 0.4 mM Ca vehicle.

Activation of the CaR affects a variety of signaling pathways in parathyroid cells (Brown & MacLeod 2001, Hofer & Brown 2003), but the pathways responsible for the control of PTH mRNA by calcium are not known. Here, we undertook pharmacologic investigation of the signaling pathways that could be involved in Ca-induced reduction of PTH mRNA, including adenyl cyclase/cyclic AMP, phospholipase D (PLD), and mitogen-activated protein (MAP) kinase. To determine the importance of cyclic AMP on regulation of PTH mRNA in the bovine pseudogland cultures, pertussis toxin (an inhibitor of adenylate cyclase formation; 50 ng/ml) or exogenous dibutyryl-cyclic AMP (2 mM) were added to the cultures. No effect was detected on PTH mRNA with either condition (Table 1). Neither selective inhibition of MAP kinase kinase (PD 98059; 50 μ M in high-calcium conditions) nor inhibition of p38/SAPK2 (SB 203580; 5 and 25 μ M in high-calcium conditions) altered PTH mRNA (Table 1). The Rho kinase and PLD signaling pathways were examined using the inhibitor Y-27632 (a selective inhibitor of Rho-associated protein kinase; 50 μ M) and *n*-butanol (which blocks the formation of phosphatidic acid; 0.5% in high-calcium conditions). These inhibitors also did not alter PTH mRNA (Table 1).

Compounds known to directly increase cytosolic Ca were used in the bovine parathyroid pseudogland model. Thapsigargin (500 nM), which increases cytosolic Ca by inhibiting the calcium pump of the endoplasmic reticulum responsible for maintaining Ca stores,

significantly decreased PTH mRNA at both low- and high-Ca conditions (Fig. 5 and Table 1). In addition, the Ca ionophore A23187 (10 μ M), significantly, and even more dramatically, decreased PTH mRNA under low-Ca conditions (not tested under high-Ca conditions), as shown in Fig. 5 and Table 1. These data indicate that cytosolic Ca regulates PTH mRNA.

Discussion

A key point in the Ca control of PTH secretion is the regulation of PTH mRNA. The parathyroid glands are programed to synthesize and secrete PTH in response to a hypocalcemic stimulus. In conditions of normal to high Ca, secretion of pre-packaged PTH is reduced and intracellular PTH is degraded. The studies of Moallem *et al.* (1998) indicated that the control of PTH mRNA by Ca does not involve the alteration in the rate of PTH gene transcription. The alternate explanation, that Ca regulates PTH mRNA stability, was supported by their *in vitro* degradation assays. Subsequent studies identified a cis element in the 3'-UTR of rat PTH that targeted reporter transcripts for degradation and that bound cytosolic proteins in a Ca-dependent manner (Moallem *et al.* 1998, Sela-Brown *et al.* 1999, 2000, Kilav *et al.* 2001, 2004, Naveh-Many *et al.* 2002); human and bovine 3'-UTR were found to have alternative cis-acting protein-binding elements involved in the stabilization of PTH mRNA (Bell

Figure 4 (A) Calcimimetic R-568 accelerates PTH mRNA decay in parathyroid cells. Bovine parathyroid pseudoglands were maintained in serum-free medium containing 0.4 mM Ca for 24 h to maximize basal secretion of PTH, and then placed in medium containing 0.4 mM Ca with or without R-568. After 2 h, the transcriptional inhibitor actinomycin D (1 µg/ml) was added; the pseudoglands were harvested for RNA at 0 or 20 h after the addition of actinomycin D. DMSO was used as vehicle. PTH mRNA and 18S rRNA were measured by northern blot analysis. Data are given as mean ± s.d. (*n* = 3 samples per condition; unpaired *t*-test analysis); **P* < 0.001 vs 0.4 mM Ca at 20 h. (B) Maximal reduction of PTH mRNA by R-568 requires gene transcription. Bovine pseudoglands were maintained in serum-free medium containing 0.4 mM Ca for 24 h to maximize basal secretion of PTH. Actinomycin D (1 µg/ml), or vehicle, was then added to block transcription. After 30 min, medium was replaced with fresh 0.4 mM Ca medium containing 10 nM R-568 ± actinomycin D. RNA was isolated from the pseudoglands 16 h later and analyzed for PTH mRNA and 18S RNA. Inhibiting gene transcription with actinomycin D prior to treatment with R-568 partially blocked the reduction of PTH mRNA by the calcimimetic, suggesting that maximal reduction of PTH mRNA by R-568 requires gene transcription (*n* = 3 samples per condition; unpaired *t*-test analysis). **P* < 0.05 versus + actinomycin.

et al. 2005a). While these studies have given valuable insight into PTH mRNA regulation, demonstration of a long-term, direct effect of Ca on PTH mRNA stability and investigation of intracellular signaling pathways in intact parathyroid cells has been hampered by the lack of an appropriate cell model. There are no parathyroid cell lines that secrete PTH in a calcium-dependent manner, and parathyroid cells lose their response to calcium within a few days of being placed in primary monolayer culture (Brown *et al.* 1995, Mithal *et al.* 1995).

In the present study, we used a three-dimensional parathyroid cell culture system developed in our laboratory, in which parathyroid cells placed in collagen form a single, tight mass of cells that is easily manipulated, and retains a stable response to calcium for several weeks (Ritter *et al.* 2004). Culturing parathyroid cells in a manner that provides a more natural three-dimensional environment is important in maintaining long-term calcium responsiveness, as shown by Ridgeway *et al.* (1986) nearly 20 years ago. In the present study, the organoids (termed pseudoglands) respond with a Ca-dependent decrease in PTH mRNA. Here, we showed that the fall in PTH mRNA occurs over the first 16 h following exposure to high Ca, and that the effect is reversed when extracellular Ca is reduced. Furthermore, we showed that increasing extracellular Ca accelerates the rate of PTH mRNA degradation in intact parathyroid cells; this observation is in agreement with the previous *in vitro* degradation assay of Moallem *et al.* (1998). The utility of the parathyroid cell pseudogland model is emphasized by our novel observation that the induction of PTH mRNA degradation by extracellular Ca requires gene transcription, an observation not possible with *in vitro* studies. The identity of the gene(s) involved in this control is currently under investigation.

The bovine parathyroid pseudoglands provide an ideal model to elucidate the signaling pathways that mediate the effect of extracellular Ca on PTH mRNA stability. The role of the CaR in Ca-regulation of PTH mRNA was confirmed using the calcimimetic R-568, which significantly decreased PTH mRNA. The effect of R-568 on PTH mRNA was shown to be due, in part, to the

Table 1 Roles of signaling pathways in regulation of parathyroid hormone (PTH) mRNA

Compound		Effect on PTH mRNA
Pathway		
Cyclic AMP	Pertussis toxin (inhibitor of G _i)	None
Cyclic AMP	Dibutyl-cyclic AMP	None
MAP kinase	PD 98059 (MEK inhibitor)	None
MAP kinase	SB 203580 (p38 inhibitor)	None
Rho kinase	Y27632 (inhibitor)	None
Phospholipase D	<i>n</i> -Butanol (blocks phosphatidic acid formation)	None
Cytosolic calcium	Thapsigargin	↓ at LC and HC
Cytosolic calcium	A23187 (calcium ionophore)	↓ at LC (N/D at high calcium)

Figure 5 Increased cytosolic calcium decreases PTH mRNA in parathyroid cells. Parathyroid pseudogland cultures were placed in 0.4 mM Ca for 24 h to maximize basal secretion of PTH. Thapsigargin (500 nM), which increases cytosolic Ca by inhibiting the calcium pump of the endoplasmic reticulum responsible for maintaining Ca stores, was added 30 min prior to challenge with 3.0 mM Ca; PTH mRNA was measured after 24 h. The Ca ionophore A23187 (10 μ M) was tested only in low-Ca conditions (0.4 mM Ca). Data are given as mean $\pm$ s.d. ($n=3$ samples per condition; unpaired t -test analysis); * $P<0.001$ versus respective vehicle control.

acceleration of transcript degradation. This demonstrated, for the first time, a direct effect of the calcimimetic R-568 on PTH mRNA in intact parathyroid cells, and supports the finding of *Levi et al.* (2006) who showed that the calcimimetic decreased PTH mRNA *in vivo* in rats with adenine-induced chronic renal failure, and that this effect was post-transcriptional. In addition, we found that maximal reduction of PTH mRNA by R-568 requires gene transcription.

Activation of the CaR affects a variety of signaling pathways in parathyroid cells, including adenylyl cyclase/cyclic AMP, phospholipase C (PLC), PLD, phospholipase A₂ (PLA₂), and MAP kinase (*Brown & MacLeod 2001*, *Hofer & Brown 2003*), but the pathways responsible for the control of PTH mRNA by calcium are not known. Activation of the CaR inhibits adenylyl cyclase via G_i and blocks accumulation of cyclic AMP (*Chen et al. 1989*) in bovine parathyroid cells. However, in our bovine parathyroid pseudogland system, inhibiting adenylyl cyclase formation or adding exogenous dibutyryl-cyclic AMP had no effect on PTH mRNA. Activation of MAP kinase pathways in response to calcium also occurs in the parathyroid gland (*Kifor et al. 2001*); however, in the present studies, a specific inhibitor of the MEK pathway and an inhibitor of the p38 had no effect on PTH mRNA.

Additionally, inhibiting the Rho kinase and PLD signaling pathways appeared to have no effect on PTH mRNA in our culture system.

It is known that increases in extracellular Ca lead to an elevation of cytosolic Ca in parathyroid cells (*Nemeth & Scarpa 1987*, *Muff et al. 1988*). However, despite the implication that an increase in cytosolic Ca controls PTH secretion, it has been shown that the regulation of PTH secretion can be dissociated from the increase in cytosolic Ca (*Nemeth & Scarpa 1986*). Consequently, the exact mechanism of regulation of PTH secretion by calcium is still in question. Moreover, the mechanism of Ca regulation of PTH gene expression is also not fully known. While others have shown *in vivo* that hypercalcemia decreases levels of PTH mRNA (*Yamamoto et al. 1989*), and *in vitro* in cultured bovine parathyroid cells that high extracellular Ca can decrease PTH mRNA (*Russell et al. 1983*, *Brookman et al. 1986*), demonstration of the direct regulation of PTH mRNA by increased cytosolic Ca has never been shown. Therefore, in the present study, compounds known to directly increase cytosolic Ca were used in the bovine parathyroid pseudogland model. Thapsigargin (500 nM), which increases cytosolic Ca by inhibiting the calcium pump of the endoplasmic reticulum responsible for maintaining

Ca stores, significantly decreased PTH mRNA at both low- and high-Ca conditions. In addition, the Ca ionophore A23187 (10 μ M) decreased PTH mRNA even more dramatically under low-Ca conditions (not tested under high-Ca conditions). Therefore, an increase in cytosolic Ca is implicated in the regulation of PTH mRNA.

One of the best-known mediators of the effects of cytosolic Ca is the Ca-binding protein calmodulin (CaM). Formation of the Ca:CaM complex activates CaM-dependent protein kinases and the protein phosphatase calcineurin. Calcineurin has been implicated in the regulation of basal levels of PTH gene expression (Bell *et al.* 2005b). PTH mRNA levels were increased, though still regulated by low-Ca and phosphorus diets, in mice with genetic deletion of the calcineurin $\text{A}\beta$ gene. This signaling pathway is currently under investigation in our bovine parathyroid pseudogland cell model.

In conclusion, using our three-dimensional culture system that retains a stable response to Ca, we were able to demonstrate for the first time a reversible, time-dependent Ca regulation of PTH mRNA in intact parathyroid cells. In addition, extracellular Ca induces a destabilization of PTH mRNA decay of PTH mRNA that requires gene transcription. Examination of intracellular signaling pathways indicates that the Ca-mediated decrease in PTH mRNA involves an increase in intracellular Ca.

Acknowledgements

This research was supported by Amgen and National Institutes of Health Grant DK-53774. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Bell O, Silver J & Naveh-Many T 2005a Identification and characterization of cis-acting elements in the human and bovine PTH mRNA 3'-untranslated region. *Journal of Bone and Mineral Research* **20** 858–866.
- Bell O, Gaberman E, Kilav R, Levi R, Cox KB, Molkentin JD, Silver J & Naveh-Many T 2005b The protein phosphatase calcineurin determines basal parathyroid hormone gene expression. *Molecular Endocrinology* **19** 516–526.
- Brookman JJ, Farrow SM, Nicholson L, O'Riordan JL & Hendy GN 1986 Regulation by calcium of parathyroid hormone mRNA in cultured parathyroid tissue. *Journal of Bone and Mineral Research* **1** 529–537.
- Brown EM & MacLeod RJ 2001 Extracellular calcium sensing and extracellular calcium signaling. *Physiological Reviews* **81** 239–297.
- Brown EM, Gamba G, Riccardi D, Lombardi D, Butters RR, Kifor O, Sun A, Hediger M, Lytton J & Hebert SC 1993 Cloning and characterization of an extracellular Ca^{2+} -sensing receptor from bovine parathyroid. *Nature* **366** 575–580.
- Brown AJ, Zhong M, Ritter C, Brown EM & Slatopolsky E 1995 Loss of calcium responsiveness in cultured bovine parathyroid cells is associated with decreased calcium receptor expression. *Biochemical and Biophysical Research Communications* **212** 861–867.
- Chattopadhyay N, Mithal A & Brown EM 1996 The calcium-sensing receptor – a window into the physiology and pathophysiology of mineral ion metabolism. *Endocrine Reviews* **17** 289–307.
- Chen RA & Goodman WG 2004 Role of the calcium-sensing receptor in parathyroid gland physiology. *American Journal of Physiology* **286** F1005–F1011.
- Chen CJ, Barnett JV, Congo DA & Brown EM 1989 Divalent cations suppress 3',5'-adenosine monophosphate accumulation by stimulating a pertussis toxin-sensitive guanine nucleotide-binding protein in cultured bovine parathyroid cells. *Endocrinology* **124** 233–239.
- Fukugawa M & Kurokawa K 2002 Calcium homeostasis and imbalance. *Nephron* **92** 41–45.
- Habener JF, Kemper B & Potts JT 1975 Calcium-dependent intracellular degradation of parathyroid hormone: a possible mechanism for the regulation of hormone stores. *Endocrinology* **97** 431–441.
- Hofer AM & Brown EM 2003 Extracellular calcium sensing and signaling. *Nature Reviews. Molecular Cell Biology* **4** 530–538.
- Kifor O, MacLeod RJ, Diaz R, Bai M, Yamaguchi T, Yao T, Kifor I & Brown EM 2001 Regulation of MAP kinase by calcium-sensing receptor in bovine parathyroid cells and CaR-transfected HEK-293 cells. *American Journal of Physiology* **280** F291–F302.
- Kilav R, Silver J & Naveh-Many T 2001 A conserved cis-acting element in the parathyroid hormone 3'-untranslated region is sufficient for regulation of RNA stability by calcium and phosphate. *Journal of Biological Chemistry* **276** 8727–8733.
- Kilav R, Bell O, Le SY, Silver J & Naveh-Many T 2004 The parathyroid hormone mRNA 3'-untranslated region AU-rich element is an unstructured functional element. *Journal of Biological Chemistry* **279** 2109–2116.
- Levi R, Ben-Dov IZ, Lavi-Moshayoff V, Dinur M, Martin D, Naveh-Many T & Silver J 2006 Increased parathyroid hormone gene expression in secondary hyperparathyroidism of experimental uremia is reversed by calcimimetics: correlation with posttranslational modification of the trans acting factor AUFI. *Journal of the American Society of Nephrology* **17** 107–112.
- Mithal A, Kifor O, Kifor I, Vassilev P, Butters R, Krapcho K, Simin R, Fuller F, Hebert SC & Brown EM 1995 The reduced responsiveness of cultured bovine parathyroid cells to extracellular Ca^{2+} is associated with marked reduction in the expression of extracellular Ca^{2+} -sensing receptor messenger ribonucleic acid and protein. *Endocrinology* **136** 3087–3092.
- Moallam E, Kilav R, Silver J & Naveh-Many T 1998 RNA-protein binding and post-transcriptional regulation of parathyroid hormone gene expression by calcium and phosphate. *Journal of Biological Chemistry* **273** 5253–5259.
- Muff R, Nemeth EF, Haller-Brem S & Fischer JA 1988 Regulation of hormone secretion and cytosolic Ca^{2+} by extracellular Ca^{2+} in parathyroid cells and C-cells: role of voltage-sensitive Ca^{2+} channels. *Archives of Biochemistry and Biophysics* **265** 128–135.
- Naveh-Many T, Bell O, Silver J & Kilav R 2002 Cis and trans acting factors in the regulation of parathyroid hormone (PTH) mRNA stability by calcium and phosphate. *FEBS Letters* **529** 60–64.
- Nemeth EF & Scarpa A 1986 Cytosolic Ca^{2+} and the regulation of secretion in parathyroid cells. *FEBS Letters* **203** 15–19.
- Nemeth EF & Scarpa A 1987 Rapid mobilization of cellular Ca^{2+} in bovine parathyroid cells evoked by extracellular divalent cations. Evidence for a cell surface calcium receptor. *Journal of Biological Chemistry* **262** 5188–5196.
- Ridgeway RD, Hamilton JW & MacGregor RR 1986 Characteristics of bovine parathyroid cell organoids in culture. *In Vitro Cellular and Developmental Biology* **22** 91–99.
- Ritter CS, Slatopolsky E, Santoro S & Brown AJ 2004 Parathyroid cells cultured in collagen matrix retain calcium responsiveness: importance of three-dimensional tissue architecture. *Journal of Bone and Mineral Research* **19** 491–498.

- Russell J, Lettieri D & Sherwood LM 1983 Direct regulation by calcium of cytoplasmic messenger ribonucleic acid coding for pre-parathyroid hormone in isolated bovine parathyroid cells. *Journal of Clinical Investigation* **72** 1851–1855.
- Sela-Brown A, Naveh-Mani T & Silver J 1999 Transcriptional and post-transcriptional regulation of PTH gene expression by vitamin D, calcium and phosphate. *Mineral and Electrolyte Metabolism* **25** 342–344.
- Sela-Brown A, Silver J, Brewer G & Naveh-Mani T 2000 Identification of AUF1 as a parathyroid hormone mRNA 3'-untranslated region-binding protein that determines parathyroid hormone mRNA stability. *Journal of Biological Chemistry* **275** 7424–7429.
- Yamamoto M, Igarashi T, Muramatsu M, Fukagawa M, Motokura T & Ogata E 1989 Hypocalcemia increases and hypercalcemia decreases the steady-state level of parathyroid hormone messenger RNA in the rat. *Journal of Clinical Investigation* **83** 1053–1056.

Received in final form 10 October 2007

Accepted 15 November 2007

Made available online as an Accepted Preprint
15 November 2007