

Parathyroid hormone (PTH) secretion, PTH mRNA and calcium-sensing receptor mRNA expression in equine parathyroid cells, and effects of interleukin (IL)-1, IL-6, and tumor necrosis factor- α on equine parathyroid cell function

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Abstract

Parathyroid hormone (PTH) is secreted by the chief cells of the parathyroid gland in response to changes in ionized calcium (Ca^{2+}) concentrations. In this study, we measured PTH secretion, and PTH mRNA and calcium-sensing receptor (CaR) mRNA expression by equine parathyroid chief cells *in vitro*. We also evaluated the effects of interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α on PTH secretion, and PTH and CaR mRNA expression. The relationship between PTH and Ca^{2+} was inversely related. PTH secretion decreased from 100% (day 0) to 13% (day 30). PTH mRNA expression declined from 100% (day 0) to 25% (day 30). CaR mRNA decreased from 100% (day 0) to 16% (day 30). Chief cells exposed to high (2.0 mM) Ca^{2+} concentrations had a lower PTH mRNA expression compared with low Ca^{2+} concentrations. Ca^{2+} concentrations had no effect on CaR mRNA expression. The inhibitory effect of high Ca^{2+} concentrations on PTH secretion also declined over time. After day 10, there was no significant difference in PTH secretion between low and high Ca^{2+} concentrations. IL-1 β decreased both PTH secretion (75%) and PTH mRNA expression (73%), and resulted in a significant overexpression of CaR mRNA (up to 142%). The effects of IL-1 β were blocked by an IL-1 receptor antagonist. IL-1 β decreased the Ca^{2+} set-point from 1.4 mM to 1.2 mM. IL-6 decreased PTH secretion (74%), but had no effect on PTH and CaR mRNA expression. TNF- α had no effect on PTH secretion, and PTH and CaR mRNA expression. In summary, the decreased responsiveness of parathyroid cells to Ca^{2+} from 0 to 30 days can be explained, in part, by the reduced CaR expression. IL-1 β and IL-6 but not TNF- α affected parathyroid function *in vitro* and may be important in influencing PTH secretion in the septic horse.

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Introduction

Parathyroid hormone (PTH) is secreted by the chief cells of the parathyroid gland, and plays an important role in calcium homeostasis. PTH secretion is altered by small physiological changes in extracellular ionized calcium (Ca^{2+}) concentrations, and there is an inverse sigmoidal relationship between serum Ca^{2+} concentrations and PTH secretion (Mayer & Hurst 1978, Brown 1983, Brent *et al.* 1988, Aguilera-Tejero *et al.* 1996), represented by a four-parameter model (Brown

1983), which enables the parathyroid chief cells to rapidly respond to hypocalcemia. These changes in Ca^{2+} concentrations are detected by a calcium-sensing system which includes a G protein-linked calcium receptor (CaR) and gp330/megalin/LRP-2 (a glycoprotein of the low density lipoprotein-receptor superfamily) (Brown *et al.* 1993, Lundgren *et al.* 1994, Hjalm *et al.* 1996).

In vitro studies on parathyroid cells from different species have improved our understanding of the physiology and pathophysiology of the parathyroid gland (Brown *et al.* 1976, Sakaguchi *et al.* 1987,

Fasciotta *et al.* 1989, Liu *et al.* 2001). However, no *in vitro* study on equine parathyroid cells has been reported. Horses have unique features with regard to calcium metabolism, including high serum total and ionized calcium concentrations compared with other species (Toribio *et al.* 2001), poorly regulated intestinal absorption of calcium (Schryver *et al.* 1974), high urinary fractional clearance of calcium (Toribio *et al.* 2001), low serum concentrations of vitamin D (1,25-dihydroxyvitamin D and 25-hydroxyvitamin D) (Maenpaa *et al.* 1988, Breidenbach *et al.* 1998), and a high Ca^{2+} set-point (Toribio *et al.* 2003).

Conditions of the horse characterized by abnormal parathyroid gland function include idiopathic hypocalcemia of foals (Beyer *et al.* 1997), primary hyperparathyroidism (Frank *et al.* 1998), nutritional secondary hyperparathyroidism (Ronen *et al.* 1992), humoral hypercalcemia of malignancy (Marr *et al.* 1989), hypoparathyroidism (Couetil *et al.* 1998), vitamin D toxicity (Harrington & Page 1983), renal disease (Elfers *et al.* 1986), exercise-induced hypocalcemia (Aguilera-Tejero *et al.* 2001), and sepsis (Toribio *et al.* 2001).

Hypocalcemia is a common finding in humans and horses with sepsis (Carlstedt *et al.* 1998, Toribio *et al.* 2001). In a previous study, we found that some horses with sepsis and hypocalcemia had low serum PTH concentrations for their degree of hypocalcemia, indicating an inappropriate response of the parathyroid gland to low serum Ca^{2+} concentrations (Toribio *et al.* 2001). We believe that this abnormal parathyroid gland function may have resulted, in part, from increased serum concentrations of inflammatory mediators.

Inflammatory mediators known to be increased in horses with sepsis, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)- α (Seethanathan *et al.* 1990, Barton *et al.* 1998, Bueno *et al.* 1999) have been shown in other species to decrease PTH secretion and increase the expression of the CaR mRNA in parathyroid cells and in renal tubular cells (Nielsen *et al.* 1997, Carlstedt *et al.* 1999, Canaff & Hendy 2002). Because sepsis in horses is often associated with increased concentrations of IL-1, IL-6, and TNF- α , we believe that these inflammatory mediators may directly or indirectly affect PTH secretion.

The objectives of this study were (1) to determine the functionality (measured as PTH secretion) of equine parathyroid chief cells to changes in

extracellular Ca^{2+} concentrations, (2) to measure PTH secretion, and PTH and CaR mRNA expression in equine chief cells for 30 days, and (3) to assess the effect of IL-1 β , IL-6, and TNF- α on PTH secretion, and PTH mRNA and CaR mRNA expression.

Materials and methods

Preparation of parathyroid cells

This study was performed in compliance with The Ohio State University Institutional Laboratory Animal Care and Use Committee. Equine parathyroid glands were obtained within 15 min of euthanasia from horses donated to The Ohio State University College of Veterinary Medicine for humane destruction. The glands were transported in ice-cold Dulbecco's modified Eagles' medium (DMEM)/F12 containing penicillin (100 IU/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and gentamicin (20 $\mu\text{g}/\text{ml}$). The time from collection to transport to the laboratory was 15 min.

The glands were trimmed of fat and connective tissue, and minced in DMEM/F12 medium, pH 7.4, containing Ca^{2+} (1.0 mM), Mg^{2+} (0.5 mM), and 0.5% bovine serum albumin (BSA). Dissociated cells were obtained by collagenase P (2 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN, USA) and DNase I (50 $\mu\text{g}/\text{ml}$; Sigma, St Louis, MO, USA) digestion of the minced tissue in DMEM/F12 medium, pH 7.4, containing penicillin (10 IU/ml), streptomycin (10 $\mu\text{g}/\text{ml}$), gentamicin (4 $\mu\text{g}/\text{ml}$), Ca^{2+} (1.0 mM), Mg^{2+} (0.5 mM), and 0.5% BSA, in a 5% CO_2 incubator (Forma Scientific Inc., Marietta, OH, USA) for 45–60 min at 37 °C, with continuous shaking (100 r.p.m.), and vigorous pipetting every 15 min using a 60 cc syringe. The cell suspension was then filtered through a 100 μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA), and sedimented twice by centrifugation at 100 **g** for 5 min to reduce cellular debris and fibroblast contamination. Cell viability after processing, assessed by trypan blue dye exclusion, was estimated to be around 95%.

The cells were subsequently washed in DMEM/F12 medium supplemented with 0.5% BSA. The final parathyroid culture medium was DMEM/F12, pH 7.4, supplemented with penicillin (10 IU/ml), streptomycin (10 $\mu\text{g}/\text{ml}$), gentamicin

(4 µg/ml), $1 \times \text{ITS}^+$ premix consisting of insulin, transferrin, selenous acid, BSA, and linoleic acid (BD Discovery Labware, Bedford, MA, USA), Mg^{2+} (0.5 mM), and variable concentrations of Ca^{2+} (0.5–3.0 mM). To maintain the parathyroid cells in suspension we replaced fetal bovine serum (FBS) with ITS because, based on preliminary experiments from our laboratory using 5–10% FBS and from a report (Racke & Nemeth 1993), the absence of FBS in the medium resulted in decreased attachment of the parathyroid cells to the culture plates, making their collection easier, although the cells remain responsive to Ca^{2+} (Racke & Nemeth 1993). We used low concentrations of streptomycin and gentamicin (polycations) to reduce intracellular Ca^{2+} mobilization, which inhibits PTH secretion (Katz *et al.* 1992).

The Ca^{2+} /PTH relationship *in vitro* – the Ca^{2+} set-point

The effect of varying Ca^{2+} concentrations on PTH secretion was evaluated on day 0 (collection day) using parathyroid cells in suspension. The cells (1×10^4 cells) were incubated at 37 °C, 5% CO_2 , for 3 h in parathyroid culture medium ($\text{Ca}^{2+} = 0.5\text{--}3.0$ mM) on a shaker at 100 r.p.m. This experiment was performed three times ($n=3$). At the end of the incubation time and after centrifugation at 500 g for 5 min, the medium was aspirated and frozen at –20 °C until analysis for intact PTH, and the cells were lysed to determine cell protein concentration. PTH production was expressed as % of maximal PTH concentration (PTH_{max}) measured, and normalized to cell protein content.

The Ca^{2+} set-point was calculated as the Ca^{2+} concentration at which intact PTH concentration was 50% of PTH_{max} .

PTH secretion and PTH and CaR mRNA expression

The effect of time on PTH secretion was determined in 1×10^4 cells incubated in a low Ca^{2+} concentration (0.8 mM) medium for 3 h on days 0, 1, 2, 5, 10, 15, 20, 25, and 30. This experiment was performed three times ($n=3$). At the end of the incubation time, and after centrifugation at 500 g for 5 min, the medium was aspirated and frozen at –20 °C until analysis for intact PTH, and the cells were lysed to determine protein concentration.

The effect of time on PTH and CaR mRNA expression was determined in 0.5×10^6 parathyroid chief cells ($\text{Ca}^{2+} = 0.8$ mM) on days 0, 1, 2, 5, 10, 15, 20, 25, 30, and expressed as PTH mRNA/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA or CaR mRNA/GAPDH mRNA ratio. Controls consisted of PTH mRNA or CaR mRNA/GAPDH mRNA expression on day 0 (100% expression). This experiment was performed twice ($n=2$).

The effect of low (0.8 mM) and high (2.0 mM) Ca^{2+} concentrations on CaR and PTH mRNA expression was evaluated by Northern blot analysis in 0.5×10^6 chief cells on days 1 and 2. This experiment was repeated twice ($n=2$).

The effect of varying Ca^{2+} concentrations (0.8–2.0 mM) on PTH secretion was determined in parathyroid chief cells (1×10^4 cells) incubated for 3 h on days 0, 5, 10, 15, and 30. PTH concentrations were normalized for protein content, and expressed as % of controls ($\text{Ca}^{2+} = 0.8$ mM) for each day. This experiment was repeated three times ($n=3$).

Effect of IL-1 β , IL-6, and TNF- α on PTH secretion, and PTH and CaR mRNA expression

The effect of recombinant human IL-1 β (20, 200, and 2000 pg/ml), IL-6 (0.5, 1, 5, and 10 ng/ml), and TNF- α (0.5, 1, 5, and 10 ng/ml) (R&D Systems Inc., Minneapolis, MN, USA) on PTH secretion, and PTH and CaR mRNA expression was examined in parathyroid chief cells (0.5×10^6) for 24 h in a low Ca^{2+} (0.8 mM) medium. This experiment was performed twice ($n=2$). The concentrations of IL-1 β , IL-6, and TNF- α used in this study were chosen from values in the literature (Nielsen *et al.* 1997, Carlstedt *et al.* 1999, Canaff & Hendy 2002) and from values measured in blood samples from diseased horses. Equine cells are biologically responsive to recombinant human IL-1, IL-6, and TNF- α (Frean *et al.* 2000, Richardson & Dodge 2000).

To determine if the effect of IL-1 β on PTH secretion, and PTH and CaR mRNA expression was mediated through an IL-1-specific receptor, a recombinant human IL-1 receptor antagonist (IL-1ra; Amgen Inc., Thousand Oaks, CA, USA) at 100 ng/ml was added to the medium ($\text{Ca}^{2+} = 0.8$ mM) 2 h previous to the addition of IL-1 β (2000 pg/ml). The cells were incubated for

24 h after adding IL-1 β . In addition, parathyroid chief cells were also incubated with IL-1ra alone (100 ng/ml).

Effect of IL-1 β on the Ca²⁺ set-point

To determine the effect of IL-1 β on the Ca²⁺ set-point, parathyroid cells (5×10^4) were incubated with IL-1 β (2000 pg/ml) or without IL-1 β (controls) for 24 h with different Ca²⁺ concentrations (0.5–2.0 mM). The Ca²⁺ set-point was calculated as the Ca²⁺ concentration at which intact PTH concentration was 50% of PTH_{max}. This experiment was repeated three times ($n=3$).

The effect of IL-6 and TNF- α on the Ca²⁺ set-point was not evaluated in this study.

PTH, CaR, and GAPDH mRNA expression

To measure PTH mRNA expression, a 399 base pair canine probe with 95% homology to equine PTH was generated by polymerase chain amplification of canine PTH cDNA (Rosol *et al.* 1995) (primers: 5'-GTGTGTGAAGATGATGTCTGC-3'; 5'-TTGTTGCCCTATGCTGTCTA-3'). For CaR mRNA expression, a 1.6 kb CaR cDNA was generated by RT-PCR (primers: 5'-TGGAAGTGGGTGGGCACAAT-3', 5'-CCAGCAGGAAGTGCAGGTTGAG-3') of equine parathyroid gland total RNA, subcloned into pCR4-TOPO (Invitrogen, Carlsbad, CA, USA), sequenced, and used as a probe. For GAPDH mRNA expression, we used a 780 bp fragment of human GAPDH cDNA with 90% homology to equine GAPDH.

The probes were labeled with [³²P]dATP (Perkin Elmer/NEN Life Science Products, Boston, MA, USA) using a random primers DNA labeling system (Invitrogen) to 1×10^9 c.p.m./ μ g DNA.

Total RNA was extracted from 0.5×10^6 parathyroid chief cells using guanidinium thiocyanate and silica-based filters (RNAqueous-4 PCR kit; Ambion, Austin, TX, USA). Total RNA (10 μ g) was electrophoresed on denaturing formaldehyde (6%) agarose (1%) gels. After electrophoresis for 3 h at 70 V, the RNA was transferred onto nylon membranes (Stratagene, La Jolla, CA, USA). The membranes were UV crosslinked (Crosslinker 1800; Stratagene), prehybridized (Ultrasorb solution; Ambion) for 1 h at 42 °C and hybridized in Ultrasorb solution with the denatured herring sperm DNA and the cDNA probe (1×10^6 c.p.m./ml

hybridization solution) for 16 h. After hybridization, the membranes were washed twice for 5 min in $2 \times$ SCC/0.1% SDS at room temperature and twice with $0.1 \times$ SCC/0.1% SDS at 42 °C for 15 min. Quantitation of mRNA was performed using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA, USA), and ImageQuant software (Molecular Dynamics). The cDNA probes were stripped from the membranes by boiling twice in $0.1 \times$ SCC/0.1 SDS for 10 min. The same membranes were sequentially hybridized with the PTH, CaR, and GAPDH probes.

Measurement of PTH

Intact PTH was measured with a two-site immunochemiluminometric assay (Immulite intact PTH assay; Diagnostic Products Corporation, Los Angeles, CA, USA), specific for human intact PTH, and previously validated to measure equine serum intact PTH concentrations (Toribio *et al.* 2001). PTH concentrations were normalized to cell protein content.

Protein determination

Cell protein was extracted from the parathyroid cells using RIPA cell lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton \times 100, 1% sodium deoxycholate, 1% SDS, and 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitors (10 mg/ml leupeptin and 10 mg/ml aprotinin). Protein concentrations were measured with the Coomassie brilliant blue binding protein assay using BSA as the protein standard.

Electrolyte measurements

Electrolytes (Ca²⁺, Mg²⁺, Na⁺, K⁺) and pH in the parathyroid culture medium were measured using a NOVA 8 analyzer (Nova Biomedical, Waltham, MA, USA).

Statistical analyses

Results are presented as means \pm s.d. Experiments were repeated two or three times, and each repetition represented an independent sample. Comparisons between groups were made using the *t*-test or the Mann-Whitney rank test. Comparisons

with Tukey's test for multiple comparisons or the Kruskal–Wallis one-way ANOVA with Dunn's test for multiple comparisons depended on the data distribution (Fisher & Van Belle 1993). When analyzing the effect of time and different Ca^{2+} concentrations on PTH secretion, and PTH and CaR mRNA expression, two-way ANOVA with Tukey's test for multiple comparisons was used.

The Ca^{2+} set-point was defined as the Ca^{2+} concentration at which intact PTH concentration was 50% of PTH_{max} (Felsenfeld & Llach 1993) and determined using a four-parameter curve-fitting sigmoidal model (Toribio *et al.* 2003).

In the four-parameter model,

$$y = \frac{a-d}{1 + \left(\frac{x}{c}\right)^b} + d$$

y = PTH released, a = maximum PTH concentration measured (PTH_{max}), d = minimum PTH concentration measured (PTH_{min}), x = Ca^{2+} concentration, c = Ca^{2+} set-point, and b = slope.

To evaluate the effect of time on PTH suppression, a Ca^{2+} set-point was determined for each repetition on days 0 and 5. Subsequently, the mean \pm S.D. for the Ca^{2+} set-point of day 0 and day 5 was calculated. For parathyroid cells treated with IL-1 β , the Ca^{2+} set-point was determined using the same method. The Ca^{2+} set-points were calculated using the equation editing function of SigmaPlot 8.0 (SPSS Inc., Chicago, IL, USA). Statistical analyses were performed with SigmaStat 2.01 for Windows (SPSS Inc.). P values less than 0.05 were considered statistically significant.

Results

The Ca^{2+} /PTH relationship *in vitro* – the Ca^{2+} set-point

When equine parathyroid chief cells in suspension were exposed to varying Ca^{2+} concentrations for 3 h, the relationship between PTH and Ca^{2+} concentrations was sigmoidal (Fig. 1). PTH secretion ranged from 455 ± 102 ng/mg cell protein ($\text{Ca}^{2+} = 0.8$ mM, 100% PTH secretion) to 55 ± 27 ng/mg cell protein ($\text{Ca}^{2+} = 3.0$ mM, 12% PTH secretion).

Ca^{2+} concentrations greater than 2.0 mM did not result in an additional decrease in PTH

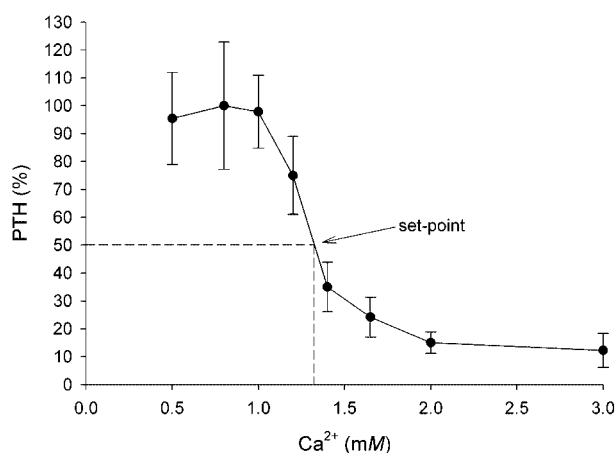


Figure 1 Equine parathyroid chief cells on day 0 (collection day) were exposed to different Ca^{2+} concentrations for 3 h. Values are means \pm S.D., $n=3$. A sigmoidal relationship between PTH and Ca^{2+} concentrations was displayed. Calcium concentrations greater than 2.0 mM did not result in an additional decrease in PTH secretion, and Ca^{2+} concentrations lower than 0.8 mM did not result in additional secretion of PTH. The Ca^{2+} set-point, calculated as the Ca^{2+} concentration at which PTH concentration was 50% of PTH_{max} , was 1.30 mM.

secretion, and Ca^{2+} concentrations lower than 0.8 mM did not result in additional secretion of PTH. Subsequent experiments were performed with Ca^{2+} concentrations of 0.8–2.0 mM.

The Ca^{2+} set-point in fresh parathyroid cells in suspension, calculated as the Ca^{2+} concentration at which PTH concentration was 50% of PTH_{max} , was 1.30 ± 0.05 mM (Fig. 1), which is similar to the Ca^{2+} set-point that we have previously determined in healthy horses (1.37 ± 0.05 mM) (Toribio *et al.* 2003).

PTH secretion, and PTH and CaR mRNA expression

PTH secretion in a low Ca^{2+} (0.8 mM) medium decreased over time. On day 0 PTH secretion was 385 ± 52 ng/mg cell protein (100%). By day 5 of culture PTH secretion declined to $68 \pm 12\%$, by day 10 to $33 \pm 6\%$, and by day 30 to $13 \pm 3\%$ compared with that of day 0 (Fig. 2). CaR and PTH mRNA expression declined over time when compared with day 0. By day 2 of culture, CaR mRNA expression declined to $41 \pm 7\%$, by day 5 to $31 \pm 6\%$, by day 10 to $21 \pm 7\%$, and by day 30 to $16 \pm 6\%$ of controls (day 0) (Fig. 2). PTH mRNA

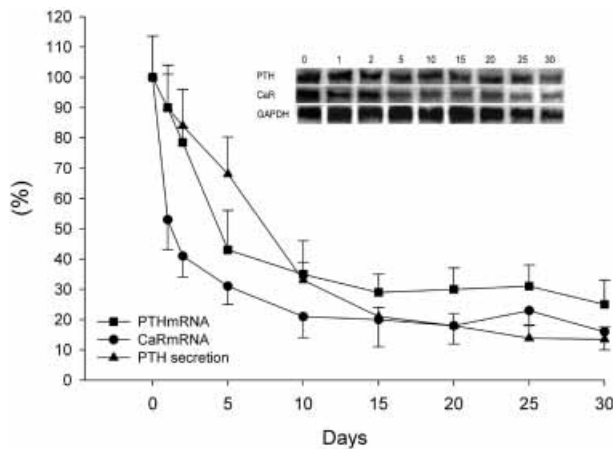


Figure 2 PTH secretion, and PTH and CaR mRNA expression in equine parathyroid cells cultured for 30 days in a low Ca^{2+} (0.8 mM) medium. Values are means \pm s.d., $n=3$. PTH secretion, PTH and CaR mRNA expression decreased over time when compared with day 0. By day 5 PTH secretion was $68 \pm 12\%$, by day 10 it was $33 \pm 6\%$, and by day 30 PTH secretion was $13 \pm 3\%$ compared with that on day 0. By day 2 of culture CaR mRNA expression decreased to 41%, by day 5 to 31%, and by day 30 to 16% of the controls (day 0). PTH mRNA expression decreased, but not as rapidly as CaR mRNA expression. By day 2 PTH mRNA expression was 78%, by day 5 it was 43%, by day 10 it was 35%, and by day 30 it was 25% of the controls.

expression declined, but not as rapidly as CaR mRNA. By day 2 PTH mRNA expression was $79 \pm 5\%$, by day 5 it was $43 \pm 13\%$, by day 10 it was $35 \pm 11\%$, and by day 30 it was $25 \pm 8\%$ of controls (day 0) (Fig. 2).

The inhibitory effect of high Ca^{2+} concentrations on PTH secretion also declined over time. By day 5 of culture, high Ca^{2+} concentrations inhibited PTH secretion; however, to obtain an equivalent PTH suppression, higher Ca^{2+} concentrations were required (Fig. 3), indicating an increase in the Ca^{2+} set-point. When the Ca^{2+} set-points for days 0 and 5 were calculated, the set-point for day 5 was higher (1.47 ± 0.1 mM) than the set-point for day 0 (1.35 ± 0.02 mM); however, this difference was not statistically significant, probably because the PTH_{\min} measured for day 5 was statistically higher ($P < 0.05$) than the PTH_{\min} for day 0. PTH_{\min} is critical in the curve-fitting model and, therefore, it affects the Ca^{2+} set-point. After day 10 of culture there was no significant difference in PTH secretion between low and high Ca^{2+} concentrations (Fig. 3).

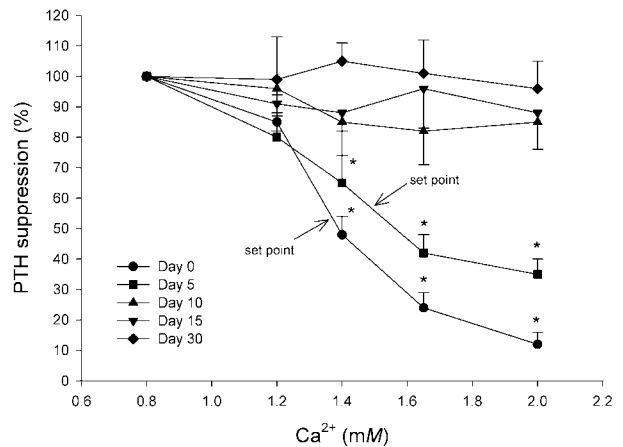


Figure 3 Effect of time on responsiveness of equine parathyroid chief cells to changes in Ca^{2+} concentrations (0.8–2.0 mM). Parathyroid cells were cultured for 24 h. Values are means \pm s.d., $n=3$. Parathyroid cells from day 0 secreted less PTH than cells from day 5 ($P < 0.05$). By day 5 of culture parathyroid cells remained responsive to changes in Ca^{2+} ; however, there was a rightward shift in the Ca^{2+} set-point from 1.35 ± 0.02 mM (day 0) to 1.47 ± 0.1 mM (day 5) because higher Ca^{2+} concentrations were required to inhibit PTH secretion. This rightward shift in the Ca^{2+} set-point was not statistically significant. After day 10 of culture there was no significant difference in PTH secretion between low and high Ca^{2+} concentrations. Control (100% PTH secretion) for each day was the PTH secreted at a Ca^{2+} concentration of 0.8 mM. * $P < 0.05$.

When the effect of low (0.8 mM) and high (2.0 mM) Ca^{2+} concentrations on CaR mRNA expression was evaluated on days 1 and 2, no effect of low (0.8 mM) or high (2.0 mM) Ca^{2+} concentrations on CaR mRNA expression was detected (Fig. 4). There was a decline in CaR mRNA expression that was time dependent, but was not statistically associated with Ca^{2+} concentrations. PTH mRNA expression on day 1 was not significantly different between low and high Ca^{2+} concentration experiments; however, on day 2, parathyroid chief cells exposed to high Ca^{2+} concentrations had lower PTH mRNA expression than cells exposed to low Ca^{2+} concentrations ($P < 0.05$) (Fig. 4).

Effect of IL-1 β , IL-6, and TNF- α on PTH secretion, and PTH and CaR mRNA expression

When parathyroid cells were incubated with increasing concentrations of IL-1 β , an IL-1 β

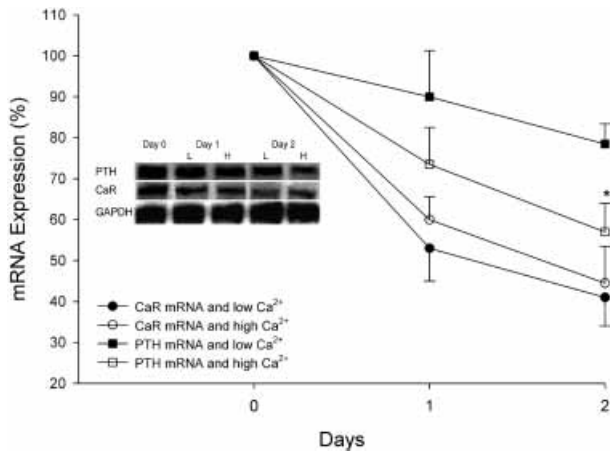


Figure 4 Effect of low (L, 0.8 mM) and high (H, 2.0 mM) Ca^{2+} concentrations on CaR mRNA and PTH mRNA expression in equine parathyroid cells. Values are means \pm S.D., $n=2$. No effect of low or high Ca^{2+} concentrations on CaR mRNA expression was detected on either day 1 or day 2. The decline of CaR mRNA was time dependent and not statistically associated with Ca^{2+} concentrations. No significant difference between low and high Ca^{2+} concentrations and PTH mRNA expression was found on day 1. However, on day 2, parathyroid cells exposed to high Ca^{2+} concentrations had significantly lower PTH mRNA expression than cells exposed to low Ca^{2+} concentrations. * $P<0.05$.

concentration of 2000 pg/ml decreased both PTH secretion (75%) and PTH mRNA expression (73%) ($P<0.05$) (Fig. 5). No statistically significant differences in PTH secretion and PTH mRNA expression at lower IL-1 β concentrations were detected when compared with controls; however, a trend for reduced PTH secretion and mRNA expression was present at 200 pg/ml IL-1 β (Fig. 5). In contrast, IL-1 β concentrations greater than 200 pg/ml resulted in a significant ($P<0.05$) overexpression of CaR mRNA (up to 142%). When IL-1ra was added to the culture medium 2 h prior to adding IL-1 β (2000 pg/ml), there was no significant difference in PTH secretion, and PTH and CaR mRNA expression when compared with controls (no IL-1 β), suggesting that the effects of IL-1 β on PTH secretion, and PTH and CaR mRNA expression were mediated by an IL-1-specific receptor. No effect of IL-1ra on PTH secretion, and PTH and CaR mRNA expression was detected when compared with controls.

IL-6 (5 and 10 ng/ml) decreased PTH secretion (74%), but had no effect on PTH and CaR mRNA expression (Fig. 6). We found no effect of TNF- α

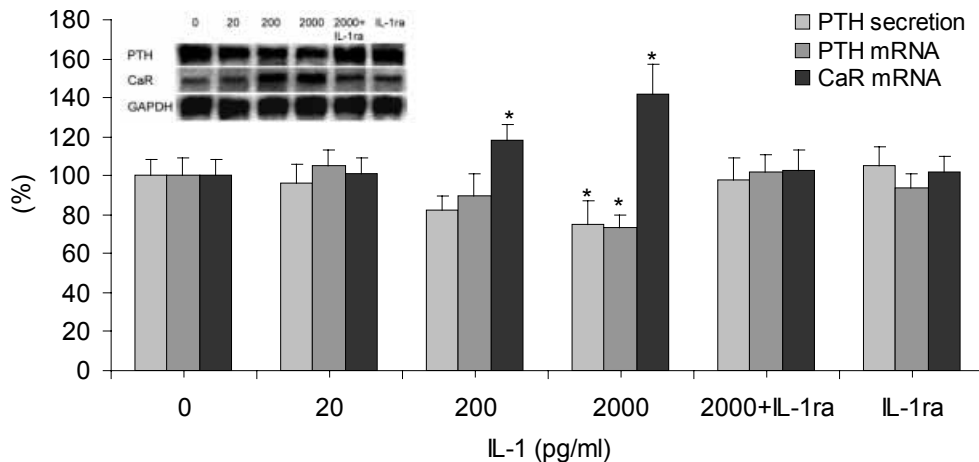


Figure 5 Equine parathyroid chief cells incubated with human recombinant IL-1 β and human recombinant IL-1ra (24 h). Values are means \pm S.D., $n=2$. Parathyroid cells incubated with IL-1 β (2000 pg/ml) had a significant decrease in both PTH secretion (75%) and PTH mRNA expression (73%). No significant difference in PTH secretion and PTH mRNA expression was detected at lower IL-1 β concentrations; however, a trend for low PTH secretion and PTH mRNA expression was present. IL-1 β concentrations greater than 200 pg/ml resulted in a significant overexpression of CaR mRNA (up to 142%). The effects of IL-1 β on PTH secretion, and PTH mRNA and CaR mRNA expression were blocked when IL-1ra (100 ng/ml) was added to the culture medium 2 h prior to IL-1 β . No effect of IL-1ra on PTH secretion, and PTH and CaR mRNA was detected when compared with controls. * $P<0.05$.

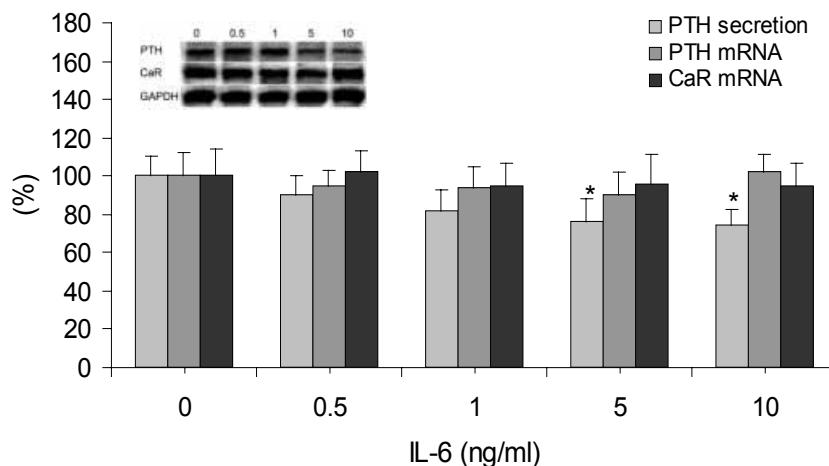


Figure 6 Effect of recombinant human IL-6 on PTH secretion, and PTH and CaR mRNA expression in equine parathyroid cells (24 h). Values are means \pm s.d., $n=2$. IL-6 concentrations higher than 5 ng/ml significantly decreased PTH secretion but had no effect on PTH and CaR mRNA expression. * $P<0.05$.

on PTH secretion, and PTH and CaR mRNA expression (Fig. 7). Due to the lack of effect of TNF- α on parathyroid cell function, we evaluated the biological activity of the TNF- α used in this study with WEHI-164 clone 13 fibrosarcoma cells (kindly donated by Dr Maxey Wellman, The Ohio State University) for which TNF- α is cytotoxic (Espevik & Nissen-Meyer 1986). Results of the cytotoxicity assay indicated that the

batch of TNF- α used in our study was biologically active.

Effect of IL-1 β on the Ca²⁺ set-point

When parathyroid cells were incubated with IL-1 β (2000 pg/ml) and various Ca²⁺ concentrations (0.8–2.0 mM) for 24 h, we found that there was a decrease in the Ca²⁺ set-point from

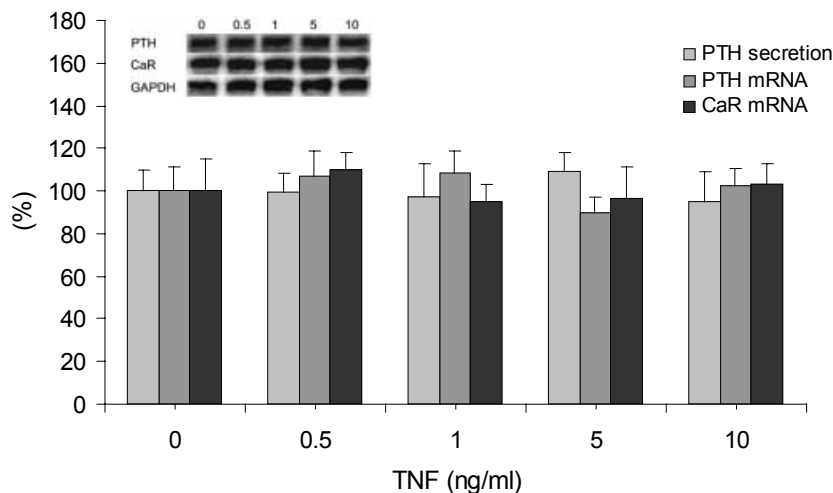


Figure 7 Effect of recombinant human TNF- α on PTH secretion, and PTH and CaR mRNA expression in equine parathyroid cells (24 h). Values are means \pm s.d., $n=2$. No effect of TNF- α on PTH secretion, PTH and CaR mRNA expression was found.

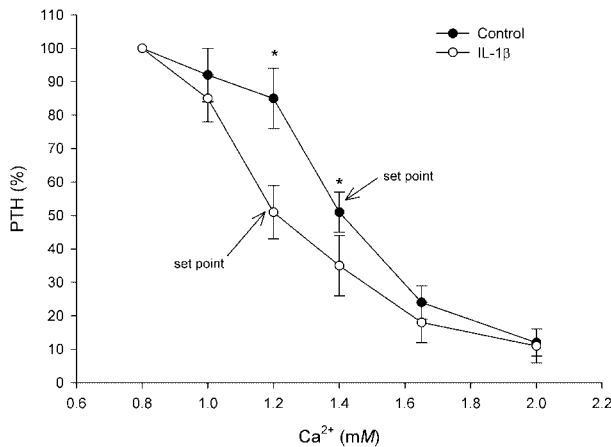


Figure 8 Effect of IL-1 β on equine parathyroid cell Ca²⁺ set-point. Values are means \pm s.d., $n=3$. Incubation of parathyroid cells with IL-1 β (2000 pg/ml) decreased PTH secretion and shifted the Ca²⁺ set-point to the left from 1.4 mM to 1.2 mM. * $P<0.05$.

1.40 \pm 0.05 mM (control) to 1.20 \pm 0.05 mM (IL-1 β treated) ($P<0.05$; Fig. 8).

Discussion

In this study, we have demonstrated that equine parathyroid chief cells cultured *in vitro* were responsive to changes in extracellular Ca²⁺ concentrations for up to 5 days after collection. However, higher Ca²⁺ concentrations were required to inhibit PTH secretion over time. These findings suggest that equine parathyroid chief cells must be used early after collection when evaluating their responsiveness to changes in extracellular Ca²⁺ concentrations. Similar findings were reported with bovine parathyroid cells, in which there was decreased parathyroid cell responsiveness (PTH secretion) to changes in Ca²⁺ concentrations over time (Mithal *et al.* 1995).

Equine parathyroid cells had a rapid decrease in CaR mRNA expression *in vitro* and, by day 5, CaR mRNA expression was reduced to 31%. We speculate that the decreased sensitivity of equine parathyroid cells to high extracellular Ca²⁺ concentrations resulted, in part, from decreased CaR mRNA expression and protein synthesis, as previously reported in bovine parathyroid cells (Mithal *et al.* 1995). Mithal *et al.* (1995) demonstrated that, over time, bovine parathyroid chief cells in culture had decreased expression of CaR

mRNA, and decreased immunostaining for the extracellular domain of CaR. By day 4 of culture, CaR mRNA expression decreased to 21% and CaR immunostaining decreased to 8–18% in bovine chief cells. It is important to note that, despite the low expression of CaR mRNA (day 5), the cells were still responsive to changes in extracellular Ca²⁺ concentrations, although PTH secretion on day 5 was 68% and PTH mRNA expression was 43% of day 0. We speculate that even with a 70% reduction in CaR mRNA expression, there was sufficient CaR protein to suppress PTH secretion, or possibly the megalin/gp330/LRP-2 calcium-sensing protein (or other calcium-sensing mechanisms) may be regulating PTH secretion in these cells (Lundgren *et al.* 1994, Hjalm *et al.* 1996). Decreased mRNA expression and protein synthesis for megalin/gp330/LRP-2 may contribute to determining the Ca²⁺ set-point (Farnebo *et al.* 1998). CaR protein synthesis was not evaluated in this study, although it would have been expected to be low, as previously reported (Mithal *et al.* 1995).

PTH mRNA expression decreased over time, but not as rapidly as CaR mRNA expression, suggesting that CaR expression may be more critical for PTH secretion in equine parathyroid chief cells in culture compared with PTH mRNA expression. There was a decrease in parathyroid cell sensitivity to Ca²⁺ concentrations, which we believe resulted from the decreased expression of CaR mRNA and an increase in the Ca²⁺ set-point, as previously reported (Kifor *et al.* 1996). We found that the Ca²⁺ set-point on day 5 was higher than the set-point for day 0; however, we could not demonstrate statistical differences, probably because the PTH_{min} for day 5 was significantly higher than the PTH_{min} for day 0.

It is unclear why there was a decrease in CaR mRNA expression in equine chief cells, but we consider that the lack of growth factors (and calcitriol) may have resulted in parathyroid cell dedifferentiation, and subsequently in decreased calcium-sensing ability (Brown *et al.* 1996, Nielsen *et al.* 1997).

No effect of changes in medium Ca²⁺ concentrations on CaR mRNA expression was found. These results are similar to those of Brown *et al.* (1996) who found that CaR mRNA was not regulated by Ca²⁺ concentrations, but rather by calcitriol. The effect of calcitriol on CaR mRNA

and PTH mRNA expression was not evaluated in this study. We also determined that PTH mRNA expression was inversely related to Ca^{2+} concentrations; PTH mRNA expression was significantly lower on day 2 in high Ca^{2+} concentration medium as compared with low Ca^{2+} concentration medium. In corroborating experiments, Moallem *et al.* (1998) found that hypocalcemia (and hyperphosphatemia) increased PTH mRNA stability by changing the ability of cytosolic proteins to bind the PTH mRNA 3'-untranslated region in rats. Factors not evaluated in this study that may affect PTH mRNA expression, synthesis, and secretion, include endothelin 1, PTH fragments, chromogranin A and its bioactive peptides, phosphate, vitamin D, and other calcium-sensing proteins such as megalin/gp330/LRP-2 (Fujii *et al.* 1991, Fujimi *et al.* 1991, Lundgren *et al.* 1994, Kilav *et al.* 1995, Slatopolsky & Delmez 1996, Fasciottio *et al.* 2000).

IL-1 is a cytokine with neuroendocrine, endocrine, metabolic, cardiovascular, and proinflammatory functions (Dinarello 2000). Our study showed that IL-1 β inhibits PTH secretion, decreases PTH mRNA expression, and increases CaR mRNA expression in equine parathyroid cells cultured for 24 h. These effects were blocked when parathyroid cells were incubated with an IL-1 receptor antagonist, indicating that equine parathyroid cells have specific receptors for IL-1. In a previous study with bovine parathyroid cells, Nielsen *et al.* (1997) found that the effect of IL-1 β to inhibit PTH secretion and to increase CaR expression was present in parathyroid tissue slices but not in dispersed parathyroid cells. The effect of IL-1 β on equine parathyroid tissue slices was not evaluated in the present study. Although equine parathyroid cells became less responsive to changes in extracellular Ca^{2+} over time in culture as occurs with bovine parathyroid cells (Mithal *et al.* 1995), we found that equine parathyroid cell function could still be altered by IL-1 β . Incubation of parathyroid cells with IL-1 β shifted the Ca^{2+} set-point to the left, indicating that the effects of IL-1 β on PTH secretion are the result of an increase in parathyroid cell sensitivity to extracellular Ca^{2+} concentrations, most likely from increased expression of CaR. We speculate that increased serum concentrations of IL-1 in septic horses may result in CaR mRNA overexpression, lowering the Ca^{2+} set-point, and reducing the

response of the parathyroid gland to hypocalcemia.

IL-6 is a pleiotropic cytokine that acts on a wide variety of cell types. It is important in regulating the immune system, is a mediator of the acute-phase response, and is increased during sepsis in humans and horses. Typical stimuli for IL-6 production are IL-1, TNF- α , and bacterial endotoxin (Akira *et al.* 1993). We found that IL-6 decreased PTH secretion; however, it had no effect on PTH and CaR mRNA expression, indicating that the effects of IL-6 on PTH production are post-translational by affecting synthesis, secretion, and degradation. TNF- α is also a pleiotropic cytokine that plays a major part in growth regulation, differentiation, inflammation, tumorigenesis, and autoimmune diseases (Aggarwal 2000). Although we were surprised not to find a positive or negative effect of TNF- α on PTH secretion, or in PTH and CaR mRNA expression, we do not rule out the possibility that TNF- α may be affecting equine parathyroid gland function during sepsis. A recent study evaluating the effect of IL-1 β and TNF- α on CaR transcriptional activity found that TNF- α and IL-1 β stimulated CaR transcription via nuclear factor- κ B in human renal tubular cells (Canaff & Hendy 2002). These authors speculated that these mechanisms may, in part, contribute to the hypocalcemia in critically ill human patients. Another possibility for the lack of biological activity of TNF- α on parathyroid cell function may have been that an intact parathyroid architecture was required for the parathyroid cells to elicit a response, as reported for IL-1 and phosphate (Nielsen *et al.* 1996; Nielsen *et al.* 1997). To our knowledge, the effect of TNF- α on parathyroid cell function has not been evaluated previously in any species, and further studies on the relationship between TNF- α and parathyroid gland function, in particular during sepsis and hypocalcemia, are justified.

Several conditions in the horse are associated with abnormal calcium homeostasis (Harrington & Page 1983, Elfers *et al.* 1986, Marr *et al.* 1989, Ronen *et al.* 1992, Beyer *et al.* 1997, Couetil *et al.* 1998, Frank *et al.* 1998, Aguilera-Tejero *et al.* 2001, Toribio *et al.* 2001). However, there is limited information on equine parathyroid gland function.

In conclusion, we have demonstrated that equine parathyroid chief cells cultured *in vitro* are responsive to changes in extracellular Ca^{2+} concentrations for up to 5 days. There was a time-dependent decrease in PTH secretion, and

PTH and CaR mRNA expression. Based on the rapid decrease in CaR mRNA expression, we believe that CaR mRNA expression is more critical in determining the *in vitro* responsiveness of equine parathyroid chief cells to changes in extracellular Ca^{2+} than PTH mRNA expression. We found that IL-1 β and IL-6, inflammatory mediators known to be increased in septic horses, inhibited PTH secretion and mRNA expression, and increased CaR mRNA expression. In our culture system, however, TNF- α did not affect PTH secretion or PTH and CaR mRNA expression. We consider that overexpression of CaR from the IL-1 β treatment could have increased Ca^{2+} sensitivity in the equine parathyroid chief cells, resulting in decreased PTH secretion. These findings indicate that IL-1 β may play a role in the regulation of equine parathyroid gland function *in vivo*. This is the first time that equine parathyroid cell function has been evaluated *in vitro*, and information generated from this study will be valuable for future research on calcium metabolism in the horse, and in understanding parathyroid gland function in healthy and sick horses and humans.

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