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PARATHYROID GLAND FUNCTION AND CALCIUM REGULATION IN HEALTHY AND SEPTIC HORSES

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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* * * * *

The Ohio State University
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ABSTRACT

Extracellular calcium (Ca^{2+}) concentrations are tightly regulated by complex homeostatic mechanisms, including parathyroid hormone (PTH), calcitonin, and 1,25-dihydroxyvitamin D (calcitriol). PTH is secreted by chief cells of the parathyroid gland, and is considered the most important calcium-regulating hormone. *In vitro* studies in parathyroid chief cells from different species have improved our understanding on the physiology and pathophysiology of the parathyroid gland. However, there is limited information on parathyroid gland function in horses. Horses have unique features with regard to calcium metabolism, including high serum Ca^{2+} concentrations, high urinary fractional clearance of calcium, low serum concentrations of vitamin D (1,25-dihydroxyvitamin D, 25-hydroxyvitamin D), and an increased Ca^{2+} set-point (Ca^{2+} concentration at which PTH secretion is 50% maximal). Several pathological conditions in horses are associated with abnormal calcium homeostasis. We have determined that in the horse (as occurs in other species) the Ca^{2+}/PTH relationship is sigmoidal, and the Ca^{2+} set-point is higher than in other domestic animals and man, which may, in part, explain the high serum calcium concentrations of the horse. Hypocalcemia occurs frequently in humans with sepsis. We found that sepsis (enterocolitis) in horses was associated with hypocalcemia, and that some horses did not have the expected increase in serum PTH concentrations for the degree of hypocalcemia, suggesting impaired parathyroid gland
function. When equine parathyroid cells were studied in vitro for 30 days, we determined that, over time in culture there was a decrease in PTH secretion, and in PTH and calcium-sensing receptor (CaR) mRNA expression. Furthermore, equine parathyroid cells exposed to an inflammatory mediator, interleukin-1β (IL-1β), had decreased PTH secretion and PTH mRNA expression, and increased CaR mRNA expression compared to controls, indicating that IL-1β may play a role in regulating chief cell function in the horse. We cloned, sequenced, and determined the tissue expression of calcitonin, calcitonin gene-related peptide (CGRP)-I, and CGRP-I in the horse. Information from these genes will be valuable to further study of the role of the calcitonin gene family in calcium regulation in healthy and sick horses. Calcium homeostasis is a complex process that involves many regulatory factors. Disturbances of calcium regulation are frequent in human and veterinary medicine. With our studies we have provided additional information to understand calcium regulation in horses.
Dedicated to my wife and children
I want to especially thank my co-adviser, Dr. Catherine W. Kohn, for her trust and support during my residency program in Equine Internal Medicine and my doctoral studies at The Ohio State University. I am very thankful to my co-adviser, Dr. Thomas J. Rosol, for his friendship, great ideas, and suggestions, and for changing my perspective of molecular biology and clinical research. I am also grateful to the Distinguished University Professor, Dr. Charles C. Capen, for sharing his knowledge on calcium metabolism in animals. I want to acknowledge Dr. Gustavo Leone for his expertise and technical advice in molecular genetics.

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PUBLICATIONS

Research Publications

1. Toribio RE, Kohn CW, Chew DJ, Capen CC, Rosol TJ. Cloning and sequence
   analysis of the complementary DNA for feline preproparathyroid hormone. Am J Vet Res


10. Toribio RE. Niveles de prostaglandina F$_{2a}$ medidos a través de su metabolito (PGFM) durante el período posparto en vacas cebuinas (Bos indicus) en el trópico húmedo de Costa Rica. Notas Veterinarias, 1992; 2:9–11.

FIELDS OF STUDY

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CHAPTER I

INTRODUCTION

Calcium is one of the most important minerals in the body, with diverse physiological functions such as muscle contraction, hormone secretion, enzyme activation, cell division, cell membrane stability, neuromuscular excitability, and blood coagulation (Rosol and Capen, 1997). Due to the importance of calcium in normal intracellular and extracellular processes, maintenance of a steady state concentration of calcium is extremely important.

Calcium is found in three main compartments: the skeleton, soft tissues, and the extracellular fluid. The skeleton contains approximately 99% of the total body calcium as hydroxyapatite crystals. The remaining calcium is present in the cell membrane, endoplasmic reticulum (0.9%), and in the extracellular fluid (0.1%) (Rosol and Capen, 1997). In plasma, calcium exists as a free or ionized form (50%), bound to proteins (40%), and complexed to anions (10%) (Hurwitz, 1996).

Regulation of extracellular ionized calcium (Ca\(^{2+}\)) concentrations is controlled by a complex homeostatic system that includes three major hormones: parathyroid hormone (PTH), calcitonin (CT), and 1,25-dihydroxyvitamin D (calcitriol) (Hurwitz, 1996; Mundy and Guise, 1999). Parathyroid hormone-related protein (PTHrP) is a newly discovered protein (Suva et al., 1987) that shares considerable homology with PTH, binds and
activates the PTH-1 receptor, and is important in cell growth and differentiation and in calcium transport across membranes (Wysolmerski and Stewart, 1998). However, in normal adults, under physiological conditions, PTHrP has no effect on calcium homeostasis (Mundy and Guise, 1999). Excessive secretion of PTHrP by some tumors results in humoral hypercalcemia of malignancy.

The biological functions of PTH include stimulation of osteoclastic bone resorption, thereby increasing Ca$^{2+}$ release into the blood, stimulation of calcium reabsorption and inhibition of phosphate reabsorption in the renal tubules, and stimulation of calcitriol synthesis in the kidney, which then increases intestinal calcium and phosphate absorption and inhibits PTH secretion from the parathyroid gland (Rosol and Capen, 1997).

PTH is secreted by the chief cells of the parathryoid gland in response to small changes in extracellular Ca$^{2+}$ concentrations. There is an inverse sigmoidal relationship between serum Ca$^{2+}$ concentrations and PTH secretion (Brown, 1983; Chapter 3), represented by a four-parameter model (Brown, 83), which enables the parathyroid gland to rapidly respond to changes in Ca$^{2+}$ concentrations. Changes in extracellular Ca$^{2+}$ concentrations are detected by a calcium-sensing G protein-linked cation receptor (CaR) in the parathyroid cells (Brown et al., 1993).

Abnormal calcium homeostasis frequently occurs in horses with various pathological conditions. However, our knowledge of equine calcium regulation and parathyroid gland function is limited. Horses have unique features with regard to calcium metabolism, including high serum total and ionized calcium concentrations (Toribio et al., 2001; Chapter 2), high urinary fractional clearance of calcium (Toribio et al., 2001;
Chapter 2), low serum concentrations of vitamin D (1,25-dihydroxyvitamin D, 25-hydroxyvitamin D) (Mäenpää et al., 1988; Breidenbach et al., 1998), and an increased Ca\(^{2+}\) set-point (Chapter 3). Pathological 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), pseudohyperparathyroidism (Marr et al., 1989), hypoparathyroidism (Couëtil et al., 1998), vitamin D toxicity (Harrington and Page, 1983), renal disease (Elfers et al., 1986), exercise-induced hypocalcemia (Aguilera-Tejero et al., 2001), and sepsis (Toribio et al., 2001; Chapter 2).

Hypocalcemia is a common finding in humans and horses with sepsis (Carlstedt et al., 1998; Toribio et al., 2001; Chapter 2). Hypocalcemia may result in neuromuscular, cardiovascular, and gastrointestinal tract dysfunction. Muscle weakness, tetany, synchronous diaphragmatic flutter, cardiac arrhythmias, ileus, and convulsions have been observed in hypocalcemic horses (Grubb et al., 1996). Severe hypocalcemia can also result in death. Clinical observation has revealed that hypocalcemia is common in horses with severe gastrointestinal disease (colic, enterocolitis). To determine the prevalence of hypocalcemia, and to evaluate parathyroid gland function in septic horses, we studied horses admitted to The Ohio State University College of Veterinary Medicine with a history of severe enterocolitis (diarrhea). We found that hypocalcemia is frequent in horses with severe diarrhea (80%). Furthermore, some horses with sepsis and hypocalcemia have low serum PTH concentrations for the degree of hypocalcemia, indicating an inappropriate response of the parathyroid gland to low serum Ca\(^{2+}\) concentrations (Chapter 2). We suspect that horses with inappropriate parathyroid gland response to hypocalcemia have an abnormally low Ca\(^{2+}\) set-point.
The role of ionized magnesium (Mg^{2+}) has received less attention than that of Ca^{2+} in critically ill patients. Serum Mg^{2+} concentration can influence Ca^{2+} concentration, and Mg^{2+} deficiency can result in hypocalcemia attributable to impaired PTH secretion as well as altered renal and skeletal responsiveness to PTH (Fatemi et al., 1991). No data were available regarding serum Mg^{2+} concentrations in healthy or ill horses. We determined that hypomagnesemia was frequent in horses with enterocolitis (78%) (Toribio et al., 2001; Chapter 2). We speculate that low serum Mg^{2+} concentrations in some horses with hypocalcemia may have resulted in inappropriately low PTH concentrations with respect to degree of hypocalcemia, due to impairment of PTH secretion or synthesis. Most humans with Mg^{2+} depletion and hypocalcemia have inappropriately low serum PTH concentrations (Abbott and Rude, 1993).

To better understand equine parathyroid gland function in healthy and sick horses, we evaluated the response of the parathyroid gland to changes in serum Ca^{2+} concentrations in healthy horses (Chapter 3). The Ca^{2+} set-point, defined as the Ca^{2+} concentration corresponding to 50% of maximal PTH concentrations (Felsenfeld and Llach, 1993), or the midpoint in the PTH/Ca^{2+} sigmoidal curve (Brown, 1983), is considered a measure of parathyroid gland sensitivity to changes in Ca^{2+} concentrations (Cunningham, 1996; Felsenfeld and Llach, 1993). Abnormalities of the Ca^{2+} set-point have been reported in humans with chronic renal failure (Slatopolsky and Delmez, 1996) and primary hyperparathyroidism (Schwarz et al., 1992). We found that the Ca^{2+} set-point in healthy horses was higher (1.37 mmol/L) than in dogs and humans (1.0-1.2 mmol/L) (Chapter 3). Serum total and ionized calcium concentrations in horses are greater than those in other domestic animals and humans (Kaneko et al., 1997). The high
Ca\textsuperscript{2+} set-point in the horse may result in high serum Ca\textsuperscript{2+} concentrations. We also speculate that horses are not as sensitive as other species to the suppressive effects of Ca\textsuperscript{2+} on PTH secretion, which results in a high Ca\textsuperscript{2+} set-point.

We determined that the phenomenon of hysteresis (differing PTH concentrations for one Ca\textsuperscript{2+} concentration value depending on the direction of change in Ca\textsuperscript{2+} concentration) was present in healthy horses. This finding indicates that the parathyroid gland of the horse, in addition to detect absolute serum Ca\textsuperscript{2+} concentrations, can also detect the direction of change in serum Ca\textsuperscript{2+} concentrations (Chapter 3).

*In vitro* studies of parathyroid cells from different species have improved our understanding of the physiology and pathophysiology of the parathyroid gland (Brown et al., 1976; Liu et al., 2001; Sakaguchi et al., 1987; Fasciotto et al., 1989). However, the number of studies on equine parathyroid gland function is limited (Roussel et al., 1987; Estepa et al., 1998; Aguilera-Tejero et al., 1998; Toribio et al., 2001; Chapter 2), and no study on equine parathyroid cell function *in vitro* has been reported (Chapter 4). To expand our knowledge of equine parathyroid gland physiology we evaluated *in vitro* equine parathyroid cell morphology and proliferation, PTH secretion, PTH mRNA and calcium-sensing receptor (CaR) mRNA expression. There was an inverse sigmoidal relationship between PTH secretion and Ca\textsuperscript{2+} concentrations as occurs *in vivo* (Chapter 3; Chapter 4). Over time in cell culture, parathyroid cell PTH secretion, and PTH and CaR mRNA expression decreased, and parathyroid cells became less sensitive to changes in Ca\textsuperscript{2+} concentrations (Chapter 4).

Inflammatory mediators such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-\(\alpha\) are increased in horses with sepsis (Seethanathan et al., 1990; Barton et
expression and decrease PTH secretion in bovine parathyroid cells (Nielsen et al., 1997; Carlstedt et al. 1999). When the effect of IL-1β on equine parathyroid chief cell was evaluated in vitro, there was a decrease in PTH secretion and mRNA expression, and an increase in CaR mRNA expression (Chapter 4). Increased CaR mRNA expression results in lower PTH secretion, decreasing the Ca^{2+} set-point, and may be a reason that parathyroid glands of septic horses secrete inappropriately low PTH concentrations for the degree of hypocalcemia. We believe that IL-1 plays an important role in regulating PTH secretion in the horse.

Calcitonin (CT) is a calcium-lowering hormone synthesized by the C-cells (parafollicular cells) of the thyroid gland (Copp, 1994). Studies on the calcitonin gene family have revealed alternative splicing of the primary transcripts (Rosenfeld et al., 1981; Amara et al., 1985) resulting in mRNA products for calcitonin, calcitonin gene-related peptide (CGRP)-I, and CGRP-II. CGRPs are important in a diverse array of physiological processes including inhibition of bone resorption, modulation of neurotransmitter activity, vasodilation, relaxation of smooth muscle, alteration of gastrointestinal motility, neurogenic inflammation, and anti-inflammatory activity (Brain and Cambridge, 1996). Serum calcitonin, procalcitonin, and CGRP concentrations have been reported to be increased in human patients with hypocalcemia and sepsis (Chesney et al., 1983; Sperber et al., 1990; Parida et al., 1998; Lind et al., 2000). The importance of peptides of the calcitonin gene family in the pathogenesis of hypocalcemia in septic human patients and horses is unknown. We have cloned and sequenced the cDNA encoding for equine calcitonin, CGRP-I, and CGRP-II (Chapter 5), in order to study the importance of these peptides in the pathogenesis of hypocalcemia in horses.
In our studies we describe calcium homeostasis in healthy and septic horses. In healthy horses, we determined that the relationship between serum Ca\(^{2+}\) and PTH concentrations was sigmoidal, and that the Ca\(^{2+}\) set-point was higher than in humans and dogs. We also found that the phenomenon of hysteresis in the Ca\(^{2+}\)/PTH relationship was present in healthy horses. We cultured equine parathyroid chief cells, and evaluated their function \textit{in vitro}, in order to support future studies on equine parathyroid physiology and pathophysiology. We determined that interleukin-1\(\beta\) may alter equine parathyroid cell function \textit{in vitro}. We found that hypocalcemia is frequent in septic horses, and that many horses with hypocalcemia had abnormal parathyroid gland function. We cloned 3 different products of the equine calcitonin gene family to study their role in the pathogenesis of hypocalcemia in the horse. In conclusion, the information generated from these studies will be valuable for future research on calcium metabolism in the horse, and in understanding parathyroid gland function in healthy and sick horses.


CHAPTER 2

COMPARISON OF SERUM PARATHYROID HORMONE AND IONIZED CALCIUM AND MAGNESIUM CONCENTRATIONS AND FRACTIONAL URINARY CLEARANCE OF CALCIUM AND PHOSPHORUS IN HEALTHY HORSES AND HORSES WITH ENTEROCOLITIS

Abstract

Objective—To evaluate calcium balance and parathyroid gland function in healthy horses and horses with enterocolitis and compare results of an immunocohemiluminometric assay (ICMA) with those of an immunoradiometric assay (IRMA) for determination of serum intact parathyroid hormone (PTH) concentrations in horses.

Animals—64 horses with enterocolitis and 62 healthy horses.

Procedures—Blood and urine samples for determination of serum total calcium, ionized calcium (Ca\(^{2+}\)) and magnesium (Mg\(^{2+}\)), phosphorus, BUN, total protein, creatinine, albumin, and PTH concentrations, venous PCO\(_2\) and PO\(_2\), and fractional urinary clearance of calcium (FCa) and phosphorus (FP) were collected when horses entered the study. Serum concentrations of PTH were measured in 40 horses by use of both the IRMA and ICMA.
Results—Most (48/64; 75%) horses with enterocolitis had decreased serum total calcium, \( \text{Ca}^{2+} \), and \( \text{Mg}^{2+} \) concentrations and increased phosphorus concentrations, compared with healthy horses. Serum PTH concentration was increased in most (36/51; 70.5%) horses with hypocalcemia. In addition, FCa was significantly decreased and FP significantly increased in horses with enterocolitis, compared with healthy horses. Results of ICMA were in agreement with results of IRMA.

Conclusions and Clinical Relevance—Enterocolitis in horses is often associated with hypocalcemia; 79.7% of horses in the present study had ionized hypocalcemia. Because FCa was low, it is unlikely that renal calcium loss was the cause of hypocalcemia. Serum PTH concentrations varied in horses with enterocolitis and hypocalcemia. However, we believe low PTH concentration in some hypocalcemic horses may be the result of impaired parathyroid gland function.

Introduction

The primary function of the parathyroid gland is to prevent hypocalcemia by rapid secretion of parathyroid hormone (PTH) when serum ionized calcium (\( \text{Ca}^{2+} \)) concentrations decrease (Rosol and Capen, 1996). In several species, PTH induces an acute increase in blood calcium concentration by enhancing renal reabsorption and bone release of calcium, and a chronic increase by facilitating intestinal calcium absorption (Agus et al., 1973; McSheehy and Chambers, 1986; Nemere and Norman, 1986; Hurwitz, 1996). For reasons not yet understood, serum \( \text{Ca}^{2+} \) concentrations decrease in humans and other animals with certain pathologic conditions (eg, endotoxemia, sepsis, severe burns) (Zaloga and Chernow, 1987; Zaloga et al., 1988; Nakamura et al., 1998; Murphey
 Clinical observation has revealed that many horses with severe enterocolitis are hypocalcemic. Hypocalcemia may result in neuromuscular, cardiovascular, and gastrointestinal tract dysfunction. Muscle weakness, tetany, synchronous diaphragmatic flutter, cardiac arrhythmias, ileus, and convulsions have been observed in hypocalcemic horses (Grubb et al., 1996). Severe hypocalcemia can result in death. Supplementation with calcium added to replacement fluids appears to benefit horses with enterocolitis and hypocalcemia. Although the contribution of calcium to improvement in gastrointestinal tract and cardiac function in such horses has not been defined, we believe that restoration of calcium homeostasis is an important aspect of managing horses with enterocolitis.

We hypothesized that horses with severe enterocolitis and hypocalcemia would also have high serum PTH concentrations. High PTH concentrations would result in increased renal resorption of calcium, decreased fractional urinary clearance of calcium (FCa), and increased fractional urinary clearance of phosphorus (FP) (Agus et al., 1973; Kinoshita et al., 1986; Strewler and Rosenblatt, 1995).

The role of ionized magnesium (Mg$^{2+}$) has received less attention than that of Ca$^{2+}$ in critically ill patients. In humans, serum Mg$^{2+}$ concentration can influence Ca$^{2+}$ concentration (Rosol and Capen, 1996; Rosol and Capen, 1997), and Mg$^{2+}$ deficiency can result in hypocalcemia attributable to impaired PTH secretion as well as altered renal and skeletal responsiveness to PTH (Rosol and Capen, 1996). Treatment with magnesium restores these deficiencies (Fatemi et al., 1991). 1,25-Dihydroxyvitamin D metabolism is
more sensitive to \( \text{Mg}^{2+} \) depletion than either PTH secretion or PTH-mediated osteoclastic bone resorption (Rude et al., 1985). The effects of \( \text{Mg}^{2+} \) on mineral metabolism may be more important in chronically ill patients than has been appreciated (Fatemi et al., 1991). Few data are available regarding serum \( \text{Mg}^{2+} \) concentrations in healthy or ill horses.

There are multiple radioimmunoassays for use in measuring PTH concentrations. The assays recognize either the amino-, carboxyl-, or midregion fragment of PTH or the full-length (intact) hormone (Felsenfeld and Llach, 1993). Results of radioimmunoassays for the carboxyl- and midregion fragments do not reflect biological activity of the hormone; only assays for the N-terminal region or intact hormone measure biologically active PTH (Rodriguez et al., 1991). The introduction of 2-site immunometric assays to measure intact PTH has resulted in an important advance, because these assays provide greater reliability and precision than earlier assays (Brown et al., 1987; Nussbaum et al., 1987). Two-site immunometric assays for PTH use 2 antibodies, one to the N-terminal region and the other to the carboxyl-terminal region. Intact PTH is captured between these 2 antibodies. The immunoradiometric assay (IRMA) for PTH has been shown to be useful in dogs (Torrance and Nachreiner, 1989), cats (Barber et al., 1993), cows (Yamagishi and Naito, 1997), and horses (Martin et al., 1996; Beyer et al., 1997; Estepa et al., 1998; Aguilera-Tejero et al., 1998; Frank et al., 1998). In horses, measurement of serum PTH concentration has been used to aid in the diagnosis of idiopathic hypocalcemia of foals (Beyer et al., 1997), primary hyperparathyroidism (Roussel et al., 1987; Peauroi et al., 1998; Frank et al., 1998), nutritional secondary hyperparathyroidism (Ronen et al., 1992; Clarke et al., 1996), pseudohyperparathyroidism (Roussel et al., 1987; Marr et al., 1989), hypoparathyroidism (Couëtil et al., 1998), vitamin D toxicosis
serum PTH concentration has also been used to investigate the relationship between serum Ca\(^{2+}\) and PTH concentrations in healthy horses (Roussel et al., 1987; Martin et al., 1996; Estepa et al., 1998; Aguilera-Tejero et al., 1998).

Recently, an automated solid-phase 2-site immunochromiluminometric assay (ICMA) for quantitative determination of intact PTH has become available. Results of this assay and those of the IRMA are in good agreement for human serum (Chen and Tsai, 1996; Michelangeli et al., 1997). However, the ICMA can detect lower serum PTH concentrations and is more sensitive and more reliable than the IRMA (Michelangeli et al., 1997).

The purposes of the study reported here were to compare the ICMA and IRMA for measurement of intact PTH in horses and to evaluate calcium balance and parathyroid gland function in healthy horses and horses with enterocolitis by measuring serum total calcium and Ca\(^{2+}\), phosphorus, Mg\(^{2+}\), and intact PTH concentrations and FCa and FP.

Materials and Methods

Animals—This study was approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. The affected group consisted of 64 (39 sexually intact females, 18 castrated males, 7 sexually intact males) horses with enterocolitis that were selected over a 1-year period from horses admitted to The Ohio State University Veterinary Teaching Hospital with a history of severe diarrhea of < 48 h duration. Horses in this group were between 1 and 22 years of age (6.3±5.1 years) and comprised several
breeds (19 Quarterhorses, 11 Thoroughbreds, 11 Standardbreds, and 23 other breeds). Affected horses had received no calcium and no more than 5 L of fluid, administered IV or PO, prior to admission. Complete physical examinations, including cardiovascular, respiratory tract, and gastrointestinal tract assessment and assessment of rectal temperature, hydration status, and mentation, were performed immediately after admission. Clinical evidence of acute, profuse, watery diarrhea and dehydration were required for entry into the study.

The control group consisted of 62 (42 sexually intact females, 14 castrated males, 6 sexually intact males) healthy horses between 2 and 21 years of age (mean ± SD, 6.9 ± 3.6 years). Healthy horses were selected from the Ohio State University College of Veterinary Medicine teaching herd during a 1-year period and comprised several breeds (35 Standardbreds, 15 Thoroughbreds, and 12 other breeds). Horses in this group were fed a diet of grass and grass hay, had no history of illness for the 3 months preceding the study, and had received no treatments for the month preceding the study. Horses were determined to be healthy on the basis of results of complete physical examinations. In addition, results of CBC and biochemical analyses, including determination of serum total calcium and Ca²⁺ concentrations and plasma fibrinogen concentration, were within reference ranges.

Venous blood samples from three horses with chronic renal failure and hypercalcemia (one Thoroughbred mare of 10 years of age, one Thoroughbred gelding of 7 years of age, and one Quarter horse gelding of 10 years of age) were used to determine serum intact PTH concentrations for PTH assay validation.
Sample collection—Venous blood samples for determination of serum total calcium, \( \text{Ca}^{2+} \), total protein, albumin, creatinine, BUN, phosphorus, and \( \text{Mg}^{2+} \) concentrations were collected into tubes without additives. Venous blood samples for CBC were collected into tubes with EDTA, and for determination of plasma fibrinogen concentration, into tubes with sodium citrate. Venous blood samples were also collected under anaerobic conditions into heparinized syringes for determination of \( \text{PvCO}_2 \) and \( \text{PvO}_2 \). Urine was collected by free catch or catheterization for urinalysis and urine biochemical analyses. Blood samples for serum biochemical analyses were collected at the same time as urine.

Hematologic and biochemical analyses—Complete blood counts were performed, using an automated system.\(^a\) Serum total calcium and protein, creatinine, BUN, albumin, and phosphorus concentrations were determined by use of colorimetric reactions in an automated analyzer.\(^b\) Plasma fibrinogen concentrations were determined, using a nephelometric analyzer.\(^c\) Partial pressures of oxygen and carbon dioxide in venous blood were measured, using a blood gas analyzer,\(^d\) and pH was corrected for rectal temperature. Blood samples for \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) determinations were processed immediately, using \( \text{Ca}^{2+} \)- or \( \text{Mg}^{2+} \)-selective electrodes.\(^e\)

Fractional clearance of calcium and phosphorus—Urine was acidified with 6 N HCl to dissolve calcium salts. Urine calcium and creatinine concentrations were measured as described for serum. Fractional clearances of calcium and phosphorus were calculated, using serum and urine calcium, phosphorus, and creatinine concentrations, as described by Roussel et al. (1993).
**Determination of intact PTH concentration**—Venous blood, collected into tubes without additives, was centrifuged at 4 °C immediately after clotting, and serum was stored at −20 °C for batch analysis. Serum PTH concentration was measured by use of 2-site IRMA\(^7\) for human intact PTH that was validated for use in horses (Estepa et al., 1998) and a 2-site ICMA\(^8\) for human intact PTH.

Ten serum samples for which PTH concentrations were measured by the Nichols Institute IRMA and the ICMA (ICMA PTH range for these samples was 3.5 to 77.0 pmol/L) were selected and sent to a different laboratory\(^9\) to determine serum PTH concentrations using the 2-site DPC PTH IRMA.\(^1\) This laboratory routinely determines PTH concentration in horses using this assay and we wanted to compare their PTH results with our results within this PTH range. Intact PTH concentrations are reported in pmol/L (pmol/L = pg/ml × 0.105).

The ICMA is a solid-phase 2-site assay. The solid phase, a polystyrene bead in the test unit, is coated with an affinity-purified goat polyclonal antibody against amino acids 44 to 84 of human PTH. Serum samples and alkaline phosphatase-conjugated affinity-purified goat polyclonal antibody against amino acids 1 to 34 of human PTH were incubated for 60 min at 37 °C in the test unit,\(^1\) with intermittent agitation. Intact PTH in the sample was bound and formed an antibody sandwich complex. The assay system\(^1\) automatically handled sample and reagent additions, incubation and separation steps, and measurement of photon output by use of a temperature-controlled luminometer. Results were calculated from an observed signal, using a stored master curve (Babson, 1991). The working range was 0.1 to 263 pmol/L (1 to 2,500 pg/ml), and the sample volume was 50 µl.
The PTH IRMA (like the ICMA) is an immunometric assay based on two antibodies, in which a solid phase is coated with a high affinity-purified goat polyclonal antibody against the mid-region and C-terminal PTH. The other antibody is prepared to bind N-terminal PTH, and this antibody is labeled for detection with $^{125}$I. Only intact PTH forms the sandwich complex necessary for detection. This assay has been previously validated for intact PTH determination in horses (Estepa et al., 1998).

Sensitivity of the ICMA was defined as the smallest single value that could be distinguished from 0 at the 95% confidence limit, using equine serum. For the ICMA and IRMA, the manufacturers report a calculated sensitivity of 0.1 pmol of intact PTH/L for human serum. We determined a sensitivity of 0.12 pmol of intact PTH/L for equine serum by use of the ICMA.

The intra-assay coefficient of variation for the ICMA was determined by measuring 5 aliquots each of equine serum with low (2.5 pmol/L), medium (19.0 pmol/L), and high (180.0 pmol/L) PTH concentrations. The interassay coefficient of variation for the ICMA was determined by comparison of results of 5 replicated measures of equine serum with low (8.1 pmol/L), medium (20.5 pmol/L), and high (170.0 pmol/L) PTH concentrations. Equine serum samples with low (6.2 pmol/L), medium (26.8 pmol/L), and high (180.0 pmol/L) PTH concentrations were assayed undiluted and diluted 1:2, 1:4, and 1:8, and results were evaluated for dilutional parallelism by comparing measured (observed) values with expected (calculated) values. Two additional dilutions were performed (1:16 and 1:32) for the sample with high PTH concentration. Expected values were calculated by multiplying measured values by the dilution factor. Correlations between observed PTH and calculated PTH concentrations were determined.
Precision of the IRMA was not evaluated, because this assay has been validated for use in horses (Estepa et al., 1998).

Comparison of results between the IRMA and ICMA—Serum PTH concentrations were determined by use of both the ICMA and IRMA for 32 horses with enterocolitis, 3 horses with chronic renal failure and hypercalcemia, and 5 clinically normal horses. Results were compared between tests.

Statistical analyses—Initial analysis consisted of descriptive statistics for all variables. Data were compared by use of an unpaired t-test or the Mann-Whitney rank test between affected and healthy horses (Fisher and Van Belle, 1993). All analyses were performed with the assistance of commercially available software.

On the basis of serum Ca\(^{2+}\) concentration, horses with enterocolitis were assigned to a normocalcemic or hypocalcemic (serum Ca\(^{2+}\) concentration < 6.0 mg/dl) group. The serum Ca\(^{2+}\) concentration cutoff used to define hypocalcemia was derived from results of a previous report of Ca\(^{2+}\) concentrations in healthy horses (Kohn and Brooks, 1990), and data from healthy horses in the present study. Calcium concentrations less than the minimum limit defined by the 95% confidence interval were considered below normal. The hypocalcemic group was further divided into 3 subgroups on the basis of serum PTH concentration. Affected horses with serum PTH concentrations within reference range were considered nonresponders, whereas horses with midrange PTH concentrations were considered mid-responders and horses with high PTH concentrations, high-responders. Results were compared among these 3 subgroups by use of a Kruskal-Wallis 1-way
ANOVA on ranks, with pair-wise multiple comparisons performed by use of the Dunn method (Glantz, 1997).

For affected horses, data regarding serum PTH concentrations failed the Kolmogorov-Smirnov test for normality (Fisher and Van Belle, 1993). Therefore, Spearman rank tests for correlation (Fisher and Van Belle, 1993) were used to calculate correlation (ρ) between serum PTH concentration in affected horses and total calcium, Ca²⁺, Mg²⁺, and phosphorus concentrations and FCa and FP. A Pearson product moment test (Fisher and Van Belle, 1993) was used to calculate correlation (r) between PTH concentration and total calcium, Ca²⁺, Mg²⁺, and phosphorus concentrations and FCa and FP in healthy horses.

Because PTH concentrations in horses with enterocolitis were not normally distributed and the test for equal variance between serum PTH concentration in healthy horses and horses with enterocolitis failed, a Mann-Whitney rank sum test (Fisher and Van Belle, 1993) was used to compare PTH concentrations between healthy and affected horses. Fractional urinary clearance of calcium and FP were compared by use of a t-test between these 2 groups. For all tests, P values < 0.05 were considered significant.

Bland-Altman bias plots, in which differences between results of ICMA and IRMA for each sample were plotted against the mean of these 2 values (Bland and Altman, 1986), were used to determine agreement between the 2 PTH assays. Bias was defined as the mean difference between values obtained for each sample by the 2 different methods, and error or variability was defined as the SD of these differences.
Results

**Physical examination results**—Mean ± SD duration of enterocolitis before admission was 27.3 ± 15.4 h for the 64 affected horses in our study. Physical examination on admission revealed that heart rate was significantly higher in horses with enterocolitis (71 ± 21 beats/min), compared with healthy horses (40 ± 1 beats/min). Respiratory rate, rectal temperature, and capillary refill time were also significantly higher in horses with enterocolitis (35 ± 17 breaths/min, 38.2 ± 0.9 °C, and 3 ± 1 seconds, respectively), compared with healthy horses (16 ± 3 breaths/min, 37.6 ± 0.4 °C, and < 2 seconds, respectively).

**Serum analytes**—Serum total calcium and Ca$^{2+}$ concentrations were significantly less in horses with enterocolitis than in healthy horses (Table 2.1). Differences in the Ca$^{2+}$-to-total calcium concentration ratio (Ca$^{2+}$/tCa) were not detected between groups. Total calcium concentrations < 10.9 mg/dl were considered less than reference range, and 48 of 64 (75.0%) horses with enterocolitis had total hypocalcemia. In addition, Ca$^{2+}$ concentrations < 6.0 mg/dl were considered less than reference range, and 51 of 64 (79.7%) horses with enterocolitis had ionized hypocalcemia. Serum phosphorus concentration was significantly higher in horses with enterocolitis than healthy horses, whereas Mg$^{2+}$ concentration was significantly less. Ionized magnesium concentrations < 0.47 mmol/L were considered less than reference range; 50 of 64 (78.0%) horses with enterocolitis had ionized hypomagnesemia.
Fractional urinary clearance of calcium and phosphorus—Urine and blood samples were collected simultaneously from 20 healthy horses and 20 horses with enterocolitis and hypocalcemia. Fractional urinary clearance of calcium was significantly less and FP significantly greater in horses with enterocolitis than in healthy horses (Table 2.2). Fractional urinary clearance of calcium in all horses with enterocolitis was less than the mean FCa of the control group (Fig 2.1).

Evaluation of the ICMA—The ICMA had intra-assay coefficients of variation of 5.6, 6.3, and 7.7% for serum samples with low (2.5 pmol/L), medium (19.0 pmol/L), and high (180 pmol/L) concentrations of PTH, respectively. The interassay coefficients of variation for serum samples with low (8.1 pmol/L), medium (20.5 pmol/L), and high (170 pmol/L) PTH concentrations were 6.4, 6.3, and 5.9%, respectively. Determination of PTH concentration using serial dilutions of serum samples containing low (6.2 pmol/L), medium (26.8 pmol/L), and high (180 pmol/L) concentrations revealed parallelism between measured (observed) and expected (calculated) values ($r = 0.99$).

Comparison of the IRMA and ICMA—When results were compared by use of the Mann-Whitney test, significant differences were not detected between assays ($P = 0.80$). Results of these 2 tests were correlated ($r = 0.98$). The 2 assays were also compared by use of difference or bias plots (ie, Bland-Altman plots). Analysis of results for all serum samples suggested that differences between methods were proportionally greater as PTH concentration increased (Fig. 2.2). However, all but 3 data points were within 2 SD of the mean difference. The bias over the whole range of values was 1.9 pmol/L, and the error
(variability) was 6.9 pmol/L. When the 5 serum samples with PTH concentrations > 50 pmol/L were excluded from analysis, the bias was 0.18 pmol/L and error (variability) was 2.5 pmol/L, confirming our impression that there was proportional bias in these data. Results of the ICMA were usually greater than those of the IRMA for serum samples with high PTH concentrations. In addition, at high PTH concentrations, the difference in results between the 2 assays was greater than at lower concentrations. Our interpretation of these analyses was that, despite some proportional bias, agreement between results of ICMA and IRMA was good, and differences between values obtained by these 2 methods are not likely to be clinically relevant.

Serum intact PTH concentrations—In healthy horses (n = 62), serum PTH concentration as measured by use of the ICMA was 5.8 ± 5.7 pmol/L (median, 3.7 pmol/L; range 0.12 to 21.9 pmol/L). Horses with enterocolitis had a wide range of serum PTH concentrations (median, 29.1 pmol/L; range, 0.4 to 290 pmol/L). On the basis of serum Ca^{2+} concentration, 13 of these affected horses were further assigned to the normocalcemic group, and 51 to the hypocalcemic group.

Affected horses with ionized hypocalcemia (n = 51) were then assigned to 3 subgroups on the basis of serum intact PTH concentration (Fig 2.3). Fifteen horses were classified as nonresponders; these horses had hypocalcemia and PTH concentrations within our reference range (0.6 to 21.9 pmol/L). Twenty-six were mid-responders; horses in this group had hypocalcemia and a moderately high serum PTH concentration (range, 23.3 to 121 pmol/L). Finally 10 horses were considered high responders. These horses had high serum PTH concentrations (184 to 290 pmol/L). Significant differences in
serum total calcium, Ca\textsuperscript{2+}, phosphorus, creatinine, and BUN concentrations were not detected among these 3 subgroups (Table 2.1).

In control horses, serum Ca\textsuperscript{2+} and intact PTH concentrations were significantly \((r = -0.46)\) correlated. These values were also significantly \((p = -0.54)\) correlated in horses with enterocolitis. Serum Mg\textsuperscript{2+} concentration was significantly less in horses with enterocolitis than healthy horses.

A positive correlation between serum PTH concentration and FP was detected in healthy horses \((r = 0.52)\) and in horses with enterocolitis \((p = 0.37)\). However, no association between PTH concentration and FCa was detected in horses with enterocolitis.

Discussion

The majority \((36/51; 79.7\%)\) of horses with enterocolitis in the present study had ionized hypocalcemia; however, parathyroid gland responses to hypocalcemia were variable. Possible causes of hypocalcemia in these horses included renal loss of calcium \((Zaloga et al., 1988)\), sequestration of calcium in the lumen of the gastrointestinal tract as a result of loss or poor absorption associated with inflammation \((Nakamura et al., 1998)\), impairment in calcium mobilization \((Sperber et al., 1990; Assicot et al., 1993; Dandona et al., 1994)\), tissue sequestration of calcium \((Zaloga et al., 1988)\), and impairment of calcium release by the target tissue in response to PTH \((Zaloga et al., 1988)\).

Renal loss as a cause of hypocalcemia in horses in our study was unlikely; mean FCa was significantly less in affected horses, compared with healthy horses. Reduction in
FCa is an appropriate homeostatic response to hypocalcemia. Low FCa has also been reported in association with hypocalcemia in other species (Zaloga et al., 1988). Low urinary calcium excretion may be explained by a slow rate of calcium filtration at the glomerulus or by efficient reabsorption of filtered calcium in the renal tubules. The mechanism of renal tubular cell reabsorption of calcium in humans and other animals with hypocalcemia has not been well described but may be PTH-mediated via an increase in tubule intracellular cAMP concentration (Sibbald et al., 1977). An association between high PTH and intracellular cAMP concentrations has been described in some humans with sepsis and hypocalcemia (Sibbald et al., 1977). Alternatively, renal reabsorption of calcium may be mediated by non-PTH dependent mechanisms. It has been suggested that the cell membrane Ca\(^{2+}\)-sensing receptor plays an important role in the control of renal calcium reabsorption in the thick ascending limb of the loop of Henle via phospholipase A\(_2\), adenylate cyclase, and the sodium-potassium-chloride cotransporter (Brown et al., 1999).

Impairment of calcium mobilization may be the result of impaired parathyroid gland secretion of PTH (Taylor et al., 1978; Sibbald et al., 1978), poor osteoclast response to PTH (Zaloga et al., 1988), or high serum concentrations of calcitonin and procalcitonin (Sperber et al., 1990; Assicot et al., 1993; Dandona et al., 1994; Oberhoffer et al., 1999). Secretion of PTH may have been impaired in the nonresponder group of affected horses in the present study. Serum PTH concentrations are low in some humans with sepsis and hypocalcemia (Arnaud et al., 1971) or hypocalcemia and severe burns (Klein et al., 1977) and in sheep with burns and hypocalcemia (Murphey et al., 2000). Impaired PTH secretion in our nonresponder horses could have resulted from
upregulation of the Ca\textsuperscript{2+}-sensing receptor on parathyroid gland chief cells (Nielsen et al., 1997). Stimulation of the Ca\textsuperscript{2+}-sensing receptor results in inhibition of cellular cAMP via inhibitory G-proteins (Brown et al., 1999), and consequently, in decreased PTH secretion. Inflammatory mediators that may increase in concentration in horses with enterocolitis (e.g., interleukin-[IL-] 1, IL-6, and tumor necrosis factor-[TNF-] \( \alpha \)) may induce increased expression of Ca\textsuperscript{2+}-sensing receptors on chief cells (Nielsen et al., 1997). Endotoxin, another important mediator of enterocolitis in horses, has been shown to increase IL-1, IL-6, and TNF-\( \alpha \) concentrations in horses (Seethanathan et al., 1990; Barton et al., 1998; Bueno et al., 1999) and, therefore, may indirectly suppress PTH secretion (Nielsen et al., 1997). A diminished osteoclastic response to PTH could result in inadequate calcium mobilization from bone. High-responder horses in our study had high serum PTH concentrations but remained hypocalcemic.

Tissue sequestration as a cause of hypocalcemia has been studied in humans and other animals but was not evaluated in the present study. In humans with sepsis, intracellular calcium concentrations are high in hepatocytes, aortic smooth muscle cells, and blood cells (Hotchkiss and Karl, 1996), whereas in rats with sepsis, skeletal muscle calcium uptake is increased (Sayeed, 1996). Calcium accumulates in abdominal fluid and liver of pigs with endotoxemia (Carlstedt et al., 2000). Carlstedt et al. (2000) suggested that because interstitial fluid volume is much greater than blood volume, interstitial accumulation of calcium could result in hypocalcemia.

Several mechanisms have been suggested for increased calcium entry into cells of septic patients, including IL-1-mediated increased flux across the cell membrane (Dinarello, 1984), depletion of intracellular stores of calcium that results in a retrograde
signal and activates Ca\(^{2+}\) influx across the membrane (Hotchkiss and Karl, 1996), insulin resistance (Sayeed, 1996), and impaired Ca\(^{2+}\)-dependent ATPase activity (Massry and Fadda, 1993). It has been shown that during sepsis and endotoxemia in rats, there is an increase in cytosolic Ca\(^{2+}\) that can cause cell toxicosis and death by activation of proteases, phospholipases, and other enzymes (Song et al., 1993). We do not believe that influx of Ca\(^{2+}\) from the extracellular to the intracellular compartment in our horses can explain the observed ionized hypocalcemia, because intracellular concentrations are low (100 nM) and an increase in intracellular Ca\(^{2+}\) concentrations sufficient to disrupt cellular functions would be unlikely to cause a major change in extracellular ionized calcium concentration.

In most species, the action of vitamin D to increase calcium absorption in the proximal portion of the small intestine is an important homeostatic response to hypocalcemia. Zaloga and Chernow (1987) reported that some humans with sepsis caused by infection with Gram-negative bacteria also have renal 1\(\alpha\)-hydroxylase deficiency, vitamin D deficiency, and acquired calcitriol resistance. We did not assess serum concentrations of vitamin D in our horses with enterocolitis and hypocalcemia. Plasma concentrations of calcidiol and calcitriol are reported to be much lower in horses than most mammals and birds, and 1\(\alpha\)-hydroxylase activity in the renal cortex of horses is not detectable (Breidenbach et al., 1998). The importance of vitamin D and its metabolites in the regulation of calcium metabolism in horses requires further study.

Although hypoproteinemia and hypoalbuminemia were common findings in horses with enterocolitis, we did not detect a significant difference in Ca\(^{2+}\)/tCa between control and affected horses. This finding indicates that, overall, total calcium and Ca\(^{2+}\)
concentrations changed proportionally in the same direction in affected horses. Although a decrease in serum total calcium concentration is expected in the face of hypoalbuminemia, we did not expect that \( \text{Ca}^{2+} \) concentration would decrease as a result of low serum albumin concentration. Low \( \text{Ca}^{2+} \) concentrations in affected horses with hypoalbuminemia were likely the result of impaired calcium homeostasis.

Serum \( \text{Mg}^{2+} \) concentration was significantly decreased in horses with enterocolitis and hypocalcemia, compared with healthy horses. Unlike calcium ion concentration, for which complex regulatory mechanisms safeguard homeostasis, regulation of the magnesium ion concentration is dependent on gastrointestinal tract absorption and renal reabsorption with little endocrine control (Kayne and Lee, 1993). The low serum \( \text{Mg}^{2+} \) concentrations that we observed in our affected horses may, therefore, have been the result of poor \( \text{Mg}^{2+} \) absorption from the lumen of inflamed bowel or diminished renal reabsorption of magnesium resulting in excessive urinary losses. Because we did not determine fractional urinary clearance of magnesium, we could not assess urinary magnesium losses.

The role of \( \text{Mg}^{2+} \) in critically ill patients has received less attention than that of \( \text{Ca}^{2+} \). It has been suggested that \( \text{Mg}^{2+} \) may serve as a \( \text{Ca}^{2+} \) antagonist and prevent \( \text{Ca}^{2+} \) entry into cells during sepsis or endotoxemia (Salem et al., 1995). Magnesium may also have a protective effect in endotoxemia, because magnesium deficiency predisposes humans with endotoxemia to a poor outcome (Salem et al., 1995).

A major complication in humans with moderate to severe \( \text{Mg}^{2+} \) deficiency is hypocalcemia (Abbott and Rude, 1993). Most humans with \( \text{Mg}^{2+} \) depletion and hypocalcemia have inappropriately low serum PTH concentrations with respect to degree
of hypocalcemia, suggesting impairment in PTH secretion or synthesis. Administration of Mg\(^{2+}\) to these patients results in an increase in serum PTH concentration, whereas in clinically normal humans, Mg\(^{2+}\) administration results in a decrease in PTH concentration (Abbott and Rude, 1993). Although horses with enterocolitis in the present study had significantly lower serum Mg\(^{2+}\) concentrations than did healthy horses, serum Mg\(^{2+}\) concentrations also decreased in affected horses as PTH concentrations increased. The importance of this observation is unknown. However, some humans with Mg\(^{2+}\) deficiency and hypocalcemia have high serum PTH concentrations, suggesting end-organ resistance (renal or skeletal) to PTH (Abbott and Rude, 1993). Both renal and skeletal PTH resistance have been detected in humans with hypomagnesemia (Abbott and Rude, 1993). In addition, serum 1,25-dihydroxyvitamin D concentrations were low in humans with hypomagnesemia, suggesting that formation of vitamin D is sensitive to Mg\(^{2+}\) depletion (Rude et al., 1985). Experimentally induced Mg\(^{2+}\) depletion in humans resulted in renal resistance to PTH-induced 1,25-dihydroxyvitamin D synthesis (Fatemi et al., 1991). The role of magnesium and its interactions with calcium in horses with enterocolitis deserve further study.

The increase in FP in horses with enterocolitis and hypocalcemia was consistent with high PTH concentrations in these horses. However, the correlation between PTH concentration and FP was poor (\(\rho = 0.37\)), indicating that factors other than PTH are likely important in regulating renal phosphorus clearance in horses with enterocolitis and hypocalcemia.

In 56\% (36/64) of our horses with enterocolitis, serum Ca\(^{2+}\) concentrations were low and PTH concentrations high. However, a subgroup of these horses had low serum
concentrations of PTH despite concomitant hypocalcemia. We speculate that this low PTH response may have been attributable to suppression of PTH secretion or synthesis by inflammatory mediators such as IL-1, IL-6, and TNF-α. The mechanisms by which cytokines might alter PTH release remain to be determined. Inflammatory cytokines in horses with hypocalcemia may facilitate over expression of the Ca\(^{2+}\)-sensing receptor on parathyroid cells and permit increased concentrations of intracellular calcium, thus indirectly decreasing PTH release. Additionally, it is known that the effects of Ca\(^{2+}\) and Mg\(^{2+}\) on PTH secretion are interdependent. It is possible that hypomagnesemia in some horses may have permitted intracellular accumulation of calcium and reduced cAMP production in the parathyroid gland, resulting in decreased PTH synthesis or release. Moreover, low serum Mg\(^{2+}\) concentrations may have resulted in renal and skeletal PTH resistance.

A second subgroup of affected horses with hypocalcemia had high serum PTH concentrations (high responders). The high PTH concentrations in these horses could represent a supraphysiologic response of the parathyroid gland to the combined effects of hypocalcemia and increased concentrations of inflammatory mediators. In humans and rats, hyperphosphatemia may directly stimulate PTH release (Almaden et al., 1996; Kates et al., 1997). High PTH concentrations in the high-responder horses were associated with high serum phosphorus concentrations; mean phosphorus concentration in high-responder horses was 7.2 ± 2.5 mg/dl, whereas mean phosphorus concentrations in the mid- and nonresponders were 5.7 ± 2.8 and 5.5 ± 2.8 mg/dl, respectively. However, the difference in phosphorus concentrations among groups was not significant (P = 0.3). Serum concentrations of PTH in the high-responder group may have been high because target
tissues in these horses were resistant to PTH; hypomagnesemia may induce PTH resistance. Alternatively, high-responder horses may have had low serum concentrations of 1,25-dihydroxyvitamin D. Low 1,25-dihydroxyvitamin D concentrations appear to be permissive for enhanced PTH secretion (Kates et al., 1997).

Some (13/64; 20.3%) horses with enterocolitis had serum Ca\(^{2+}\) concentrations within reference range. We believe that in these horses, calcium homeostatic mechanisms were efficient enough to maintain extracellular Ca\(^{2+}\) concentration within the physiologic range. Another explanation might be that these horses were assessed early in the disease process. In addition, 3 affected horses with normocalcemia had serum PTH concentrations greater than the upper limit of our reference range. It is possible that this increase in serum PTH concentration was the direct result of inflammatory mediators or endotoxins acting on the parathyroid gland. Alternatively, efficient PTH release may have resulted in correction of hypocalcemia, but serum PTH concentration remained high. Nakamura et al. (1998) found that endotoxin infusion in rats resulted in an increase in serum PTH concentration in some animals.

Methods for describing agreement between results of 2 tests, such as the ICMA and the IRMA, are controversial when neither test is considered the gold standard. We included assessments of correlation and bias between the 2 tests. The Spearman correlation coefficient was high (\(\rho = 0.98\)), indicating a good association in results between tests. The Bland-Altman plot for all data revealed that bias over the whole range of values was 1.9 pmol/L, indicating that the ICMA yielded PTH concentrations 1.9 pmol/L greater than the IRMA. However, inspection of the Bland-Altman plot for all data suggests that this assessment may have overestimated the bias for low PTH.
concentrations and underestimated the bias for high concentrations (Stöckl et al., 1996).

For this reason, we examined a second Bland-Altman plot in which data were restricted to those obtained by analysis of serum samples that yielded PTH concentrations < 50 pmol/L by use of the ICMA. In the second plot, the bias was reduced to 0.2 pmol/L. Agreement between results of the 2 tests was thus better at concentrations < 50 pmol/L, and inspection of the second plot indicates that only 2 values were outside 2 SD of the mean of the differences. Serum PTH concentrations > 50 pmol/L are 2 times greater than the upper limit of our reference range for the ICMA. We believe that PTH values higher than 50 pmol/L are unlikely to provide relevant information when making clinical decisions on case management. Enhanced sensitivity, shorter incubation time, automation, and lack of radioactivity are important advantages of the ICMA over the IRMA.

Mean and reference range values for serum PTH concentrations in the healthy horses of this study, measured by use of the ICMA, (mean ± SD, 5.8 ± 5.7 pmol/L; range, 0.12 to 21.9 pmol/L) were greater than those reported by Estepa et al. (1998), measured by use of the human intact PTH IRMA (mean ± SD, 3.3 ± 2.4 pmol/L; range, 0.8 to 8.7 pmol/L) and the rat PTH IRMA (mean ± SD, 4.6 ± 3.1 pmol/L; range 1.1 to 10.1 pmol/L). Breidenbach et al. (1998), who used the human intact PTH IRMA to measure plasma PTH concentrations in horses, reported a higher mean PTH value than ours (mean ± SD, 22.9 ± 19.0 pmol/L). Serum PTH concentrations for clinically normal horses have been determined by use of C-terminal assays; values were similar to or greater than those of the present study (Roussel et al., 1987; Van Heerden et al., 1990; Enbergs et al., 1996). The PTH response of healthy horses to acute hypocalcemia was evaluated by Roussel et
al. (1987), using a C-terminal immunoassay. The reference range for PTH concentrations in that study was greater than values we determined for healthy horses in the present study. This is likely attributable to the prolonged serum half-life of the C-terminal portion of PTH (Arnaud et al., 1974). We do not recommend the use of the older C-terminal PTH assays, because they do not measure intact hormone. With the new 2-site immunometric assays for intact PTH, it is now possible to accurately determine concentrations of biologically active PTH in horses. We believe that the variation among results of previous studies is related to assay variation. Differences in the calcium and phosphorus content of diets among studies may also have contributed to these apparent differences.

Most (51/64; 79.7%) horses with enterocolitis in the present study also developed ionized hypocalcemia. The expected response of the parathyroid gland to hypocalcemia is to increase secretion of PTH. Serum PTH concentrations in some (36/51; 70.5%) of the affected hypocalcemic horses was high. However, in others (15/51; 29.5%), serum PTH concentrations did not increase in proportion to the degree of hypocalcemia. Reasons for this variation in response to hypocalcemia among horses with enterocolitis are not clear. We found that renal calcium loss (ie, FCa) was significantly lower in horses with enterocolitis than in healthy horses. We believe that impaired calcium mobilization from bone, sequestration or loss of calcium from the gastrointestinal tract, or failure of the parathyroid gland to secrete adequate PTH are the most likely causes of hypocalcemia in horses with enterocolitis.
Endnotes

*a* Coulter Counter Model S-plus, Coulter Electronics, Inc, Hialeah, Fla.

*b* Boehringer Mannheim/Hitachi 911 system, Boehringer Mannheim Corp, Indianapolis, Ind.

*c* ACL 200 Automated Coagulation Laboratory, Instrumentation Laboratory, Lexington, Mass.

*d* ABL 500, Radiometer Copenhagen, Radiometer Medical A/S, Copenhagen, Denmark.

*e* Nova 8, Nova Biomedical, Waltham, Mass.

*f* Intact PTH kit, Nichols Institute Diagnostics, San Juan Capistrano, Calif.

*g* Immulite intact PTH assay, Diagnostic Products Corporation, Los Angeles, Calif.

*h* Michigan State University Animal Health Diagnostic Laboratory, Lansing, Mich.

*i* Coat-A-Count intact PTH IRMA, Diagnostic Products Corporation, Los Angeles, Calif.

*j* Immulite System, Diagnostic Products Corporation, Los Angeles, Calif.

*k* SigmaStat 2.01 for Windows, SPSS Inc, Chicago, Ill.


Song SK, Karl IE, Ackerman JJ, Hotchkiss RS. Increased intracellular Ca²⁺: a critical link in the pathophysiology of sepsis?. *Proc Natl Acad Sci USA* 1993; 90:3933–3937.


<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy (n = 62)</th>
<th>All (n = 64)</th>
<th>Normocalcemic (n = 13)</th>
<th>Nonresponder (n = 15)</th>
<th>Mid-responder (n = 26)</th>
<th>High-responder (n = 10)</th>
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<tr>
<td>Total calcium (mg/dl)</td>
<td>11.9 ± 0.5†</td>
<td>10.08 ± 1.6</td>
<td>11.3 ± 0.9</td>
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<td>Ca²⁺ (mg/dl)</td>
<td>6.6 ± 0.3†</td>
<td>5.5 ± 0.8</td>
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<td>5.3 ± 0.4</td>
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<td>Ca²⁺/tCa</td>
<td>55.3 ± 1.8</td>
<td>55.4 ± 3.9</td>
<td>57.8 ± 6.1</td>
<td>56.2 ± 7.4</td>
<td>54.4 ± 5.1</td>
<td>53.7 ± 4.7</td>
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<tr>
<td>Mg²⁺ (mmol/L)</td>
<td>0.51 ± 0.1†</td>
<td>0.40 ± 0.1†</td>
<td>0.5 ± 0.1</td>
<td>0.45 ± 0.08</td>
<td>0.38 ± 0.1</td>
<td>0.35 ± 0.05</td>
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<tr>
<td>Phosphorus (mg/dl)</td>
<td>3.2 ± 1.0†</td>
<td>5.9 ± 2.8</td>
<td>4.0 ± 2.4</td>
<td>5.54 ± 2.8</td>
<td>5.7 ± 2.8</td>
<td>7.2 ± 2.5</td>
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<tr>
<td>PTH (pmol/L)</td>
<td>5.8 ± 5.7†</td>
<td>55.6 ± 73.5</td>
<td>12.8 ± 13.6</td>
<td>12.2 ± 5.6</td>
<td>50.6 ± 26.4</td>
<td>230.4 ± 39.6</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>14.1 ± 2.8†</td>
<td>40.8 ± 29.0</td>
<td>26.7 ± 18.2</td>
<td>36.9 ± 33.8</td>
<td>41.4 ± 25.4</td>
<td>50.2 ± 30.4</td>
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<tr>
<td>Creatinine (mg/dl)</td>
<td>1.3 ± 0.2†</td>
<td>3.6 ± 1.8</td>
<td>26.7 ± 18.2</td>
<td>3.1 ± 1.6</td>
<td>3.3 ± 1.9</td>
<td>4.6 ± 1.8</td>
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<tr>
<td>Total protein (g/dl)</td>
<td>6.9 ± 0.6†</td>
<td>5.9 ± 1.5</td>
<td>6.1 ± 1.3</td>
<td>5.8 ± 1.8</td>
<td>6.1 ± 1.4</td>
<td>6.0 ± 1.2</td>
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<tr>
<td>Albumin (g/dl)</td>
<td>3.2 ± 0.2†</td>
<td>2.7 ± 0.7</td>
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<td>pH</td>
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<td>7.34 ± 0.05</td>
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<td>Total CO2 (mEq/L)</td>
<td>26.4 ± 2.4</td>
<td>23.8 ± 4.9</td>
<td>24.2 ± 4.6</td>
<td>23.2 ± 6.2</td>
<td>25.0 ± 4.5</td>
<td>23.6 ± 4.0</td>
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</tbody>
</table>

*Horses with enterocolitis were assigned to normocalcemic and hypocalcemic groups on the basis of serum Ca²⁺ concentration (hypocalcemic, Ca²⁺ concentration < 6.0 mg/dl). Horses in the hypocalcemic group were assigned to 3 groups on the basis of serum PTH concentrations (nonresponder, PTH concentration range of 0.6 to 21.9 pmol/L; mid-responder, range of 23.3 to 121 pmol/L; high responder, range of 184 to 290 pmol/L). †Significantly (P < 0.05) different from value for all horses with enterocolitis.

Ca²⁺ = Ionized calcium. Ca²⁺/tCa = Ratio of ionized calcium to total calcium concentration. Mg²⁺ = Ionized magnesium. PTH = Parathyroid hormone.

Table 2.1—Results (mean ± SD) of serum biochemical analyses for healthy horses and horses with enterocolitis
<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy (n = 20)</th>
<th>All (n = 20)</th>
<th>Normocalcemic (n = 4)</th>
<th>Nonresponder (n = 6)</th>
<th>Mid-responder (n = 6)</th>
<th>High responder (n = 4)</th>
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</thead>
<tbody>
<tr>
<td>FCa (%)</td>
<td>3.4 ± 2.6†</td>
<td>0.8 ± 0.8</td>
<td>2.1 ± 1.8</td>
<td>0.5 ± 0.46</td>
<td>0.8 ± 0.7</td>
<td>0.3 ± 0.1</td>
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<tr>
<td>FP (%)</td>
<td>0.08 ± 0.2†</td>
<td>1.9 ± 2.6</td>
<td>1.8 ± 0.6</td>
<td>3.4 ± 3.6</td>
<td>1.0 ± 1.9</td>
<td>0.3 ± 0.0</td>
</tr>
</tbody>
</table>

See Table 2.1 for key.

Table 2.2—Mean ± SD fractional urinary clearance of calcium (FCa) and phosphorus (FP) in healthy horses and horses with enterocolitis.
Figure 2.1. Box plot of fractional urinary clearance of calcium (FCa; top) and phosphorus (FP; bottom) in healthy horses (n = 20) and horses with enterocolitis (n = 20). Mean FCa was significantly ($P < 0.05$) less and FP significantly ($P < 0.05$) greater in horses with enterocolitis. • = 5th and 95th percentiles.
Figure 2.2. Bland-Altman bias plots illustrating differences in serum intact parathyroid hormone (PTH) concentrations for 40 (top) and 35 (bottom) horses determined by use of an immunochemiluminometric assay (ICMA) and an immunoradiometric assay versus the mean of these 2 values for each sample. To obtain the plot depicted in the bottom panel, the 5 serum samples that yielded > 50 pmol of PTH/L by use of the ICMA were excluded from analysis. Notice that bias is reduced in this plot, compared with the plot depicted in the top panel. Solid top and bottom lines represent ± 2 SD.
Figure 2.3. Scatterplot of serum intact PTH concentrations versus serum ionized calcium (Ca$^{2+}$) concentrations in 62 healthy horses (○ [open circles]) and 64 horses with enterocolitis (closed symbols). On the basis of Ca$^{2+}$ and PTH concentrations, horses with enterocolitis were assigned to normocalcemic (♦ [closed diamonds]; n = 13), hypocalcemic and nonresponder (▼ [closed upside-down triangle]; n = 15), hypocalcemic and mid-responder (■ [closed squares]; n = 26), and hypocalcemic and high responder (▲ [closed triangles]; n = 10) groups. The vertical dashed line indicates the lower limit of the reference range used to determine normocalcemia.
CHAPTER 3

HYSTERESIS AND CALCIUM SET-POINT DURING STEPWISE CHANGES IN SERUM IONIZED CALCIUM IN HEALTHY HORSES

Abstract

The parathyroid gland is designed to respond to changes in extracellular ionized calcium (Ca^{2+}) concentrations by controlling parathyroid hormone (PTH) secretion. Abnormalities in Ca^{2+} homeostasis are reported in horses with several pathological conditions; however, there is little information on the regulation of calcium in horses. The objectives of the present study were to determine the Ca^{2+} set-point (the calcium concentration corresponding to 50% of maximal PTH secretion) in healthy horses, to determine whether the Ca^{2+}/PTH response curves during hypocalcemia and hypercalcemia were characterized by hysteresis (different PTH concentrations for the same serum Ca^{2+} concentration depending on the direction of changes in Ca^{2+} concentrations), and to determine if the order of experimentally-induced hypocalcemia or hypercalcemia had an effect on PTH secretion (area under the time concentration curve, AUC). The Ca^{2+} set-point and hysteresis were determined in 12 healthy horses in a crossover design by infusing Na_{2}EDTA (30-90 mg/kg/h) and calcium gluconate (4-16 mg/kg/h). Four healthy horses were infused with 0.9% NaCl to serve as controls.
The Ca\(^{2+}\) set-point was 1.37 ± 0.05 mmol/L, which is higher than values reported for humans and dogs (1.0-1.2 mmol/L). In horses in which hypercalcemia was induced first the Ca\(^{2+}\) set point was lower (1.30 ± 0.05 mmol/L). We demonstrated that the phenomenon of hysteresis was present during experimentally-induced hypocalcemia and hypercalcemia. At the same serum Ca\(^{2+}\) concentration, the serum PTH concentration was higher when the serum Ca\(^{2+}\) concentration was falling (induction of hypocalcemia and recovery from hypercalcemia) than when the serum Ca\(^{2+}\) was rising (induction of hypercalcemia and recovery from hypocalcemia). Horses in which hypocalcemia was followed by hypercalcemia secreted more (P<0.05) PTH (7440 ± 740 pmol min/L) than horses in which hypercalcemia was followed by hypocalcemia (5990 ± 570 pmol min/L).

In conclusion, this study has demonstrated that the Ca\(^{2+}\) set-point in the horse is higher than in other domestic animals and man. We have shown that the Ca\(^{2+}\)/PTH relationship in horses is sigmoidal and displays hysteresis during both hypocalcemia and hypercalcemia, and that extracellular Ca\(^{2+}\) concentrations may affect the response of the parathyroid gland to hypocalcemia. Defining the Ca\(^{2+}\) set-point in horses may help to explain the pathophysiology of hypo and hypercalcemia in this species.

Introduction

Parathyroid hormone (PTH) is secreted by the chief cells of the parathyroid gland, and plays an important role in calcium homeostasis. Parathyroid hormone 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 (Brown, 1983; Mayer and Hurst, 1978; Brent et al., 1988; Aguilera-Tejero et al., 1996). This relationship is represented by a four-parameter model (Brown, 1983), which enables the parathyroid gland to rapidly respond to hypocalcemia. The parathyroid chief cells detect changes in extracellular Ca\(^{2+}\) concentrations by a calcium-sensing G protein-linked cation receptor (CaR) (Brown et al., 1993).

The calcium set-point is defined as the serum Ca\(^{2+}\) concentration corresponding to the midpoint PTH value between maximal PTH concentration (PTH\(_{\text{max}}\)) and minimal PTH concentration (PTH\(_{\text{min}}\)) (Brown, 1983), or the Ca\(^{2+}\) concentration corresponding to 50% of PTH\(_{\text{max}}\) (Felsenfeld and Llach, 1993). The Ca\(^{2+}\) set-point is considered an indicator of the serum Ca\(^{2+}\) concentration at which PTH secretion is stimulated (Felsenfeld and Llach, 1993). Determining the Ca\(^{2+}\) set-point in the horse will provide useful information in understanding the response of the parathyroid gland when calcium homeostasis is disturbed.

Abnormalities of the Ca\(^{2+}\) set-point have been reported in humans with chronic renal failure (Slatopolsky and Delmez, 1996), hemodialysis (Pahl et al., 1996), and primary hyperparathyroidism (Schwarz et al., 1992).

Conditions of horses characterized by abnormal calcium homeostasis include idiopathic hypocalcemia of foals (Beyer et al., 1997), primary hyperparathyroidism (Frank et al., 1996), nutritional secondary hyperparathyroidism (Ronen et al., 1992), pseudohyperparathyroidism (Marr et al., 1989), hypoparathyroidism (Couëtil et al., 1998), vitamin D toxicity (Harrington and Page, 1983), renal disease (Elfers et al., 1986), and sepsis (Toribio et al., 2001).
We have reported that some horses with sepsis and ionized hypocalcemia do not have the expected increase in serum PTH concentrations for the degree of hypocalcemia (Toribio et al., 2001). Such horses may have impaired parathyroid gland function associated with an abnormally low Ca\(^{2+}\) set-point.

The Ca\(^{2+}\) set-point has been determined in humans, dogs, and horses using immunoradiometric assays (IRMA) for intact PTH (Felsenfeld and Llach, 1993; Aguilera-Tejero et al., 1996; Estepa et al., 1998).

Hysteresis in the Ca\(^{2+}\)/PTH relationship refers to the fact that, for the same serum Ca\(^{2+}\) concentration, the PTH concentration is different during the induction of either hypocalcemia or hypercalcemia than during the recovery periods (Conlin et al., 1989). Hysteresis has been demonstrated in humans (Conlin et al., 1989) and dogs (Aguilera-Tejero et al., 1996), and is thought to result from depletion of PTH stores as a consequence of prolonged or severe hypocalcemia (Schwarz et al., 1992; Kwan et al., 1993; Sanchez et al., 1996), or from the ability of the parathyroid gland to sense changes in the direction of extracellular Ca\(^{2+}\) concentrations (Aguilera-Tejero et al., 1996).

Because the parathyroid gland is designed to respond to either hypocalcemia or hypercalcemia, and there is a sigmoidal relationship between serum Ca\(^{2+}\) and PTH concentrations (Mayer and Hurst, 1978; Brown, 1983; Aguilera-Tejero et al., 1996), it is possible that the extracellular Ca\(^{2+}\) concentrations at the time of serum Ca\(^{2+}\) change may affect the response of the parathyroid gland to hypocalcemia or hypercalcemia. We speculate that PTH secretion in horses with experimentally-induced hypocalcemia followed by hypercalcemia will be different compared to PTH secretion from horses with experimentally-induced hypercalcemia followed by hypocalcemia.
The objectives of this study were 1) to determine the $\text{Ca}^{2+}$ set-point in healthy horses, 2) to determine if induction and recovery from hypocalcemia and hypercalcemia results in hysteresis of serum PTH concentration in healthy horses, and 3) to determine if the order of induction of abnormal calcium concentration (hypocalcemia followed by hypercalcemia, or hypercalcemia followed by hypocalcemia) affects PTH secretion.

**Materials and Methods**

**Horses**

Sixteen healthy horses (12 females and 4 castrated males), aged 3 to 12 (7.7 ± 2.8) years were selected from The Ohio State University College of Veterinary Medicine teaching herd. All horses were fed a diet of grass and grass hay, had no history of illnesses, and had received no treatments for one month prior to sampling. To assure health, a complete physical examination was performed. Values for a complete blood count (CBC), serum chemistry profile, serum total calcium (tCa), ionized calcium ($\text{Ca}^{2+}$), ionized magnesium ($\text{Mg}^{2+}$), PTH (1-84), phosphorus, and plasma fibrinogen concentrations, blood gases, as well as urinalysis and urine chemistry profile were within the reference ranges for all horses. The horses had experimentally-induced hypocalcemia followed by hypercalcemia, or hypercalcemia followed by hypocalcemia, and were randomly assigned to each experiment. One week later the same horses were submitted to the alternative experiment. The Ohio State University Institutional Laboratory Animal Care and Use Committee approved this study.
Induction of hypocalcemia and hypercalcemia

To induce hypocalcemia, the horses were infused with Na₂EDTA (5% solution in 5% dextrose), at a rate of 30-90 mg/kg/h. To induce hypercalcemia the horses were infused with elemental calcium (calcium gluconate 23% solution; calcium gluconate hemicalcium salt with 9.3% elemental calcium), at a rate of 4-16 mg/kg/h. The goal of the Na₂EDTA and calcium gluconate infusions was to change serum Ca²⁺ concentrations by ± 0.85 mmol/L (± 3.4 mg/dL) from the normal serum Ca²⁺ concentrations (1.65±0.1 mmol/L, 6.6 ± 0.3 mg/dL), in order to maximally stimulate or suppress PTH secretion. Na₂EDTA was infused intravenously to decrease serum Ca²⁺ concentrations to 0.8 mmol/L, and calcium gluconate was infused to increase serum Ca²⁺ concentrations to 2.5 mmol/L in 120 min. To monitor for side-effects of hypocalcemia and hypercalcemia, heart rate, respiratory rate, capillary refill time, and electrocardiogram (lead II) were evaluated every 15-20 min during the experiments.

Experiment 1. Induction of hypocalcemia followed by hypercalcemia

After baseline sampling, 6 healthy horses (4 females and 2 castrated males) were infused with Na₂EDTA at a rate of 30 mg/kg/h (time 0). The rate of infusion was increased by 10 mg/kg/h every 15 min, to a final rate of 90 mg/kg/h (90-120 min). The horses were infused with Na₂EDTA for 120 min, and then the infusion was discontinued until serum Ca²⁺ returned to normal values (recovery period). Recovery to normocalcemia was not assisted by infusion of calcium. During the recovery period,
blood Ca^{2+} concentrations were determined every 10 min until Ca^{2+} concentrations reached the reference range (1.55-1.67 mmol/L). When blood Ca^{2+} concentration was within the reference range, horses were infused with elemental calcium at a rate of 4 mg/kg/h. The infusion rate was increased by 2 mg/kg/h every 15 min to a final rate of 16 mg/kg/h (90-120 min). Elemental calcium infusion rates of 4-8 mg/kg/h are within the rates that we routinely use in critically ill hypocalcemic horses admitted to the Ohio State University College of Veterinary Medicine Equine Intensive Care Unit. Higher infusion rates of calcium gluconate were used to maximally suppress PTH secretion. After the calcium gluconate infusion was discontinued (recovery period), blood samples were taken every 10 min until blood Ca^{2+} concentrations reached the reference range, then every 4 h for 16 h to monitor serum Ca^{2+} and PTH concentrations. Blood samples were collected by venipuncture at 24 and 48 h for a CBC, a serum chemistry profile, and serum Ca^{2+} and PTH concentrations.

The time required for serum Ca^{2+} concentrations to return to the reference range after induction of either hypocalcemia or hypercalcemia was estimated as the time interval from the discontinuation of the infusion of either Na_{2}EDTA or calcium gluconate to the time when the first serum Ca^{2+} value within the reference range was documented.

Serum Ca^{2+} and PTH values obtained during the induction of hypocalcemia in this experiment were used to calculate the Ca^{2+} set-point and to model the Ca^{2+}/PTH relationship. Serum Ca^{2+} and PTH concentrations obtained during induction and recovery from hypocalcemia, and during the induction and recovery from hypercalcemia were used to determine hysteresis.

Four horses from this group were used to calculate the urinary fractional clearance of calcium (FCa) and phosphorus (FP).
**Experiment 2. Induction of hypercalcemia followed by hypocalcemia**

One week following Experiment 1, horses were submitted to experimentally-induced hypercalcemia followed by hypocalcemia. Serum chemistry profile, CBC, serum PTH, tCa, Ca\(^{2+}\), Mg\(^{2+}\), and phosphorus concentrations, as well as urine profiles and urinalyses were within the reference ranges for all horses prior to Experiment 2. Horses were infused with elemental calcium at a rate of 4 mg/kg/h (time 0) with 2 mg/kg/h increases every 15 min, to a final rate of 16 mg/kg/h (90-120 min). The horses were infused with calcium gluconate for a total of 120 min. During the recovery period, blood Ca\(^{2+}\) concentrations were measured every 10 min until Ca\(^{2+}\) concentration reached reference range values. When Ca\(^{2+}\) concentration was within the reference range, the horses were infused with Na\(_2\)EDTA at a rate of 30 mg/kg/h with 10 mg/kg/h increases every 15 min, to a final rate of 90 mg/kg/h (90-120 min). After Na\(_2\)EDTA infusion was discontinued, blood samples were taken every 10 min until Ca\(^{2+}\) reached reference range values, then every 4 h for the next 16 h to monitor serum Ca\(^{2+}\) and PTH concentrations.

Serum Ca\(^{2+}\) and PTH concentrations during induction and recovery from hypercalcemia and hypocalcemia were used to determine hysteresis, and serum Ca\(^{2+}\) and PTH values from the induction of hypercalcemia were used to model the Ca\(^{2+}\)/PTH sigmoidal relationship.
Experiment 3. Induction of hypercalcemia followed by hypocalcemia

In six additional healthy horses (4 females and 2 castrated males) experimental hypercalcemia followed by hypocalcemia was induced as described in Experiment 2. Serum Ca\(^{2+}\) and PTH concentrations from the induction and recovery from hypercalcemia and hypocalcemia were used to calculate hysteresis, and serum Ca\(^{2+}\) and PTH concentrations from the induction of hypercalcemia were used to model the Ca\(^{2+}\)/PTH sigmoidal relationship.

Four horses from this group were used to calculate FCa and FP.

Experiment 4. Induction of hypocalcemia followed by hypercalcemia

One week following Experiment 3, horses were submitted to experimentally-induced hypocalcemia followed by hypercalcemia as described in Experiment 1. Values for serum chemistry profiles, CBC, serum PTH, tCa, Ca\(^{2+}\), Mg\(^{2+}\), and phosphorus concentrations, as well as urine profiles and urinalyses were within the reference ranges prior to Experiment 4. Serum Ca\(^{2+}\) and PTH values during the induction of hypocalcemia were used to calculate the Ca\(^{2+}\) set-point and to model the Ca\(^{2+}\)/PTH relationship. Serum Ca\(^{2+}\) and PTH concentrations during the induction and recovery from hypocalcemia and hypercalcemia were used to determine hysteresis.
**Experiment 5. Control horses**

Four healthy horses were infused with 0.9% NaCl at a rate of 1 ml/kg/h for 120 min. The infusion was then discontinued for 120 min, followed by a further 120 min of infusion, and then discontinued. Blood samples were taken every 20 min from time 0 for 8 h, then every 4 h for the next 16 h to measure serum $\text{Ca}^{2+}$ and PTH concentrations. Two horses from this group were used to calculate FCa and FP.

Venous blood samples to determine serum phosphorus, total calcium, and creatinine concentrations to calculate the FCa and FP were collected every 60 min for 8 h.

For all experimental horses, the catheters were removed after the infusions were discontinued. Venous blood samples for a CBC, a serum chemistry profile, and serum $\text{tCa}$, $\text{Ca}^{2+}$ and PTH concentrations were collected by venipuncture at 24 and 48 h. Urine profiles and analyses were also performed at 24 and 48 h to assess renal function.

**Sampling**

Before the experiments, venous blood samples for serum chemistry profile, and $\text{tCa}$, $\text{Ca}^{2+}$, $\text{Mg}^{2+}$, phosphorus, and PTH concentrations were collected in tubes with no additives. Venous blood samples for a CBC and fibrinogen concentration were collected in EDTA or sodium citrate, respectively. For blood gas determinations, venous blood samples were collected anaerobically in heparinized syringes. An intravenous catheter was placed aseptically in each jugular vein. The left catheter was used for the infusion of
calcium gluconate, Na₂EDTA, or 0.9% NaCl, and the right catheter was used for blood sample collection. Catheter patency was maintained by administration of 0.9% NaCl solution at a slow rate (0.5 ml/kg/h). For serum PTH measurement, the blood samples were allowed to clot at 4 °C for one hour, centrifuged at 4 °C immediately after clotting, and stored at -20 °C until batch analysis.

Four female horses from Experiment 1, four female horses from Experiment 3, and two female horses from Experiment 5 were used to calculate FCa and FP every 60 min for 8 h. An indwelling 28 French Foley urinary catheter was left in place for 8 h. The bladder was emptied 10 min before every urine collection.

Hematology and blood analytes

Complete blood cell counts were performed by an automated system (Coulter Counter Model S-plus, Coulter Electronics, Inc, Hialeah, Florida, USA). Serum profile, tCa, and phosphorus were measured using colorimetric reactions in an automated analyzer (Boehringer Mannheim/Hitachi 911 system, Boehringer Mannheim Corp, Indianapolis, Indiana, USA). Plasma fibrinogen concentrations were determined using a nephelometric analyzer (ACL 200 Automated Coagulation Laboratory, Instrumentation Laboratory, Lexington, Massachusetts, USA). Blood gases were measured using a blood gas analysis system (ABL 500, Radiometer Copenhagen, Radiometer Medical A/S, Copenhagen, Denmark). The blood samples for Ca²⁺ and Mg²⁺ concentrations were collected under anaerobic conditions and measured immediately using Ca²⁺- or Mg²⁺-selective electrodes (Nova 8, Nova Biomedical, Waltham, Massachusetts, USA). Serum
intact PTH concentrations were determined using an immunochemiluminometric assay (Immulite intact PTH assay, Diagnostic Products Corporation, Los Angeles, California, USA) validated for the horse by our laboratory (Toribio et al., 2001).

**Fractional urinary clearance of calcium and phosphorus**

Urine was acidified with 6 N HCl to dissolve calcium salts. Urine calcium, phosphorus and creatinine concentrations were measured as described for serum. FCa and FP were calculated using serum and urine total calcium, phosphorus and creatinine concentrations as described by Roussel et al. (1993).

**The calcium set-point**

The Ca\(^{2+}\) set-point was calculated as the serum Ca\(^{2+}\) concentration at which serum PTH concentrations were 50% of maximal PTH concentration during the induction of hypocalcemia (Felsenfeld and Llach, 1993). The Ca\(^{2+}\) set-point was calculated for every animal using Ca\(^{2+}\) and PTH concentrations from the induction of hypocalcemia of Experiments 1 and 4. Values of Ca\(^{2+}\) and PTH from the recovery period after hypocalcemia were not included in the calculation of the Ca\(^{2+}\) set-point because different values of PTH were expected (hysteresis) for the same Ca\(^{2+}\) value. Because hypercalcemia may have affected the response of the parathyroid gland to hypocalcemia, we also calculated the Ca\(^{2+}\) set-point in the horses from Experiments 2 and 3.
The Ca\(^{2+}\)/PTH relationship

To determine the relationship between serum Ca\(^{2+}\) and PTH concentrations, serum Ca\(^{2+}\) and PTH concentration from the induction of hypocalcemia (Experiments 1 and 4) and the induction of hypercalcemia (Experiments 2 and 3) were used. Serum Ca\(^{2+}\) and PTH values from the recovery periods were not included because hysteresis was expected.

The Ca\(^{2+}\)/PTH relationship was determined by the four-parameter logistic model previously described by Brown (1983), and by the \(E_{\text{max}}\) sigmoidal model with a baseline value (or Hill's equation) (Gabrielsson and Weiner, 1997).

In the four-parameter logistic model,

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

\(y\)=PTH released, \(a\)=maximum PTH concentration measured during hypocalcemia (PTH\(_{\text{max}}\)), \(d\)=minimum PTH concentration measured during hypercalcemia (PTH\(_{\text{min}}\)), \(x\)=serum Ca\(^{2+}\) concentration, \(c\)=Ca\(^{2+}\) set-point, and \(b\)=slope.

In the \(E_{\text{max}}\) sigmoidal model (Hill's equation),

\[
E = E_0 + \frac{E_{\text{max}} C^n}{EC_{50}^n + C^n}
\]

\(E\)=Effect (PTH released), \(E_0\)=PTH\(_{\text{min}}\), \(E_{\text{max}}\)=PTH\(_{\text{max}}\), \(C\)=serum Ca\(^{2+}\) concentration, \(EC_{50}\)=Ca\(^{2+}\) set-point, \(n\)=slope, sigmoidicity factor, or Hill coefficient.
The serum PTH area under the time concentration curve (AUC)

To determine if the order of Na₂EDTA or calcium infusion had an effect on PTH secretion, non-compartmental analysis (linear trapezoidal method) (Gabrielsson and Weiner, 1997) was used to calculate the area under the time concentration curve (AUC) for serum PTH concentrations.

AUC \( (t_i-t_i+1) \) is the area between two time points. \( C_i \) and \( C_{i+1} \) are the corresponding serum PTH concentrations, and \( \Delta t \) is the time interval (10 min).

\[
AUC = \frac{C_i + C_{i+1}}{2} \Delta t
\]

To calculate the AUC per horse over the entire range of PTH secretion, the following formula was used,

\[
AUC = \sum_{i=1}^{n} \frac{C_i + C_{i+1}}{2} \Delta t
\]

Statistical analyses

Results were expressed as meanSD. \( PTH_{\text{max}} \) was defined as the maximal PTH concentration during Na₂EDTA-induced hypocalcemia, and \( PTH_{\text{min}} \) as the minimal PTH concentration during calcium-induced hypercalcemia. Comparisons between groups were made using the \( t \)-test or the Mann-Whitney rank test. PTH values during induction of and recovery from hypocalcemia in Experiments 1-4 were compared using the Kruskal-Wallis one-way ANOVA (Fisher and Van Belle, 1993), and multiple comparisons were
made with the Dunn's test (Fisher and Van Belle, 1993). Similarly, Kruskal-Wallis one-way ANOVA was used to compare PTH concentrations during induction of and recovery from hypercalcemia, with multiple comparisons made with the Dunn's test. Spearman rank (ρ) correlation was used to determine associations between serum Ca^{2+} and PTH concentrations during the different experiments (Fisher and Van Belle, 1993).

Hysteresis was defined as statistically different serum PTH values for the same serum Ca^{2+} value depending on the direction of change of serum Ca^{2+} concentration. P values less than 0.05 were considered significant.

Results

There were no significant differences in serum Ca^{2+} and PTH concentrations between Experiments 1 and 4, nor were there differences in the time required time required for serum Ca^{2+} concentrations to return to the reference range after induction of either hypocalcemia or hypercalcemia. Therefore, these two experiments were pooled (n=12). There were no significant differences when serum Ca^{2+} and PTH concentrations from Experiments 2 and 3 were compared, nor were there statistical differences in the time required for serum Ca^{2+} concentrations to return to the reference range after induction of hypercalcemia or hypocalcemia. Therefore, the experiments were pooled (n=12).

For calculation of the Ca^{2+} set-point and hysteresis serum PTH values were expressed as a percentage of PTH_{max} because there was heterogeneity in serum PTH concentrations between horses.
Baseline values for serum tCa, Ca^{2+}, Mg^{2+}, P, and PTH concentrations were not statistically different among experimental groups. Infusion of Na_{2}EDTA resulted in a decrease in serum Ca^{2+} concentrations and an increase in serum PTH concentrations, and infusion of calcium gluconate increased serum Ca^{2+} concentrations and decreased serum PTH concentrations (Figs. 3.1, 3.2). During hypocalcemia and hypercalcemia the correlation (p) between serum Ca^{2+} and PTH concentrations ranged from -0.5 to -0.9 (P<0.05). No significant changes in serum Ca^{2+} and PTH concentrations were present in the control horses (Fig. 3.3). Forty-eight hours after the Na_{2}EDTA and calcium gluconate infusions no abnormalities in serum chemistry profiles, CBC, urine profiles and urine analyses were detected in any horses.

**Experiments 1 and 4**

During Na_{2}EDTA-induced hypocalcemia, serum Ca^{2+} concentration decreased to 0.85 ± 0.10 mmol/L (3.5 ± 0.40 mg/dL) (normal= 1.65 ± 0.10 mmol/L, 6.6 ± 0.3 mg/dL), and serum PTH concentration increased to 53.0 ± 11.0 pmol/L (normal=4.4 ± 2.5 pmol/L) (Fig. 3.1). The mean PTH_{max} in Experiments 1 and 4 was higher than that in Experiments 2 and 3. PTH_{max} values in our experiments were greater than those reported in previous studies in horses where an immunoradiometric assay for PTH was used (Aguilera-Tejero et al., 1998; Estepa et al., 1998). After the Na_{2}EDTA infusion was discontinued, serum Ca^{2+} returned to the reference values in 106 ± 10 min (Fig. 3.1).
Using the values for 50% of PTH\textsubscript{max}, the Ca\textsuperscript{2+} set-point was calculated to be 1.37 ± 0.05 mmol/L (5.5 ± 0.3 mg/dL) (Figs. 3.4, 3.6), which is greater than the Ca\textsuperscript{2+} set-point reported for humans and dogs (Felsenfeld and Llach, 1993; Aguilera-Tejero et al., 1996).

Induction of hypocalcemia resulted in a sigmoidal relationship between serum Ca\textsuperscript{2+} and PTH concentrations (Figs. 3.4, 3.6). Maximal PTH concentrations were attained with a mild decrease in serum Ca\textsuperscript{2+} concentration, and serum Ca\textsuperscript{2+} concentrations below 1.25 mmol/L did not result in an additional increase in serum PTH concentrations. Furthermore, at serum Ca\textsuperscript{2+} concentrations below 1.10 mmol/L, serum PTH concentrations decreased in 4 horses to approximately 80% of PTH\textsubscript{max}. Hysteresis was present in the hypocalcemic phase of the experiments. For the same serum Ca\textsuperscript{2+} concentration, PTH concentrations were higher during the induction of hypocalcemia than during recovery (P<0.05), and when Na\textsubscript{2}EDTA infusion was discontinued, serum PTH concentrations decreased sharply, in a linear fashion despite the severe hypocalcemia (Fig. 3.4).

During the hypercalcemic phase of Experiments 1 and 4, serum PTH concentrations decreased to very low levels or were below the detection limit of the assay (Figs. 3.1, 3.4), and serum Ca\textsuperscript{2+} concentration increased to 2.45 ± 0.20 mmol/L (9.8 ± 0.9 mg/dL) at the end of the calcium gluconate infusion (Fig. 3.1). After the calcium gluconate infusion was discontinued, serum Ca\textsuperscript{2+} returned to baseline values in 86 ± 11 min (Fig. 3.1). For a ΔCa\textsuperscript{2+} of 0.85 mmol/L, it took approximately 20 min longer to recover from hypocalcemia than hypercalcemia. Immediately after calcium gluconate infusion was discontinued, serum PTH concentrations remained low, but once serum Ca\textsuperscript{2+} concentration was below 2.0 mmol/L serum PTH concentrations increased to greater values (P<0.05) than those during the infusion phase (hysteresis; Fig. 3.4).
During the induction of hypercalcemia, serum Ca\(^{2+}\) concentrations increased to 2.42 ± 0.20 mmol/L (9.7 ± 0.8 mg/dL), and serum PTH decreased to low concentrations or were below the limit of detection of the PTH assay (Fig. 3.2). When the calcium gluconate infusion was discontinued, serum Ca\(^{2+}\) concentrations returned to normal values in 89 ± 18 min. Serum PTH concentrations remained low when serum Ca\(^{2+}\) concentrations were greater than 2.0 mmol/L, but when serum Ca\(^{2+}\) concentrations decreased below 2.0 mmol/L, serum PTH concentrations increased to values greater (P<0.05) than those during the induction of hypercalcemia (hysteresis; Fig. 3.5). PTH concentrations of figure 3.5 are expressed as a percentage of the mean PTH\(_{\text{max}}\) from Experiments 1 and 4 (Fig. 3.4).

When serum Ca\(^{2+}\) concentration was within the reference range, hypocalcemia was induced. Serum Ca\(^{2+}\) concentration decreased to 0.83 ± 0.05 mmol/L (3.5 ± 0.2 mg/dL), and serum PTH concentration increased to 42.2 ± 8.0 pmol/L (Fig. 3.2). In contrast to the induction of hypocalcemia in Experiments 1 and 4, in which the Ca\(^{2+}\)/PTH relationship was sigmoidal, the induction of hypocalcemia in Experiments 2 and 3 resulted in a more linear Ca\(^{2+}\)/PTH relationship, and serum PTH did not reach a plateau in 8 of 12 horses (Fig. 3.5). For the same time interval and rate of Na\(_2\)EDTA infusion, induction of hypocalcemia resulted in significantly lower serum PTH concentrations in Experiments 2 and 3 than in Experiments 1 and 4 (P<0.05; Figs. 3.1-3.2, 3.4-3.5). When Na\(_2\)EDTA infusion was discontinued, serum PTH concentrations decreased in a pattern similar to that of Experiments 1 and 4, but it took longer for serum Ca\(^{2+}\) concentrations to
return to normal values (116 ± 18min) than in Experiments 1 and 4; however, the time difference was not statistically significant. For the same serum Ca\(^{2+}\) concentration, serum PTH concentrations were significantly higher during the induction of hypocalcemia and during the recovery from hypercalcemia than during recovery from hypocalcemia and induction of hypercalcemia (hysteresis; Fig. 3.5). For a \(\Delta\text{Ca}^{2+}\) of 0.85 mmol/L, it took approximately 25 min longer to recover from hypocalcemia than hypercalcemia.

Induction of hypocalcemia and hypercalcemia resulted in mild changes in serum phosphorus concentrations; however, these changes were not significantly different from baseline, nor in Experiments 1 and 4 as compared to Experiments 2 and 3.

The Ca\(^{2+}\) set-point for horses of Experiments 2 and 3 was significantly lower (1.3 ± 0.05 mmol/L, 5.2 ± 0.2 mg/dL) than the set-point for horses of Experiments 1 and 4 (P<0.05).

Serum PTH and PTH\(_{\text{max}}\) concentrations from Experiments 1 and 4 were significantly greater than serum PTH and PTH\(_{\text{max}}\) concentrations from Experiments 2 and 3 (P<0.05) during induction of hypocalcemia. However, serum PTH concentration from the recovery of hypocalcemia from Experiments 1 and 4 were not statistically different than those of Experiments 2 and 3. PTH\(_{\text{max}}\) concentrations in horses of Experiments 1 and 4 were 6 to 22-fold greater than the baseline PTH concentrations (mean was 11.3 ± 5.1-fold increase), while PTH\(_{\text{max}}\) concentrations in Experiments 2 and 3 were 5 to 12 greater than the PTH baseline concentrations (7.7 ± 2.0-fold increase).

No statistical difference in serum PTH concentrations was found during the recovery from hypercalcemia between experiments.
Experiments 5

In the control horses (Experiment 5, Fig. 3.3), there was no significant difference from baseline values in serum Ca\textsuperscript{2+} and PTH concentrations at any time point during the NaCl infusion. Serum Ca\textsuperscript{2+} and PTH concentrations remained within the reference range during the infusion of 0.9% NaCl and during the recovery period.

The relationship between serum Ca\textsuperscript{2+} and PTH concentrations

The serum Ca\textsuperscript{2+} concentration and PTH concentration (expressed as a percentage of PTH\textsubscript{max} (100%) for each horse) during the induction of hypocalcemia (Experiments 1 and 4), and the induction of hypercalcemia (Experiments 2 and 3) were plotted and analyzed by the four-parameter logistic model and the \( E_{\text{max}} \) sigmoidal model. The resulting functions were sigmoidal and almost identical (Fig. 3.6). The relationship between serum Ca\textsuperscript{2+} and PTH concentrations in the horse is therefore demonstrated to be inverse and sigmoidal as described for other species (Mayer and Hurst, 1978; Brown, 1983; Aguilera-Tejero et al., 1996). The Ca\textsuperscript{2+} set-point was 1.37 ± 0.05 mmol/L (Fig. 3.6).

The area under the PTH-time concentration curve (AUC)

When the AUC for PTH production in Experiments 1 and 4 was compared to that of Experiments 2 and 3, horses in Experiments 1 and 4 secreted more PTH (7440 ± 740
pmol min/L) than horses from Experiments 2 and 3 (5990 ± 570 pmol min/L)(P<0.05),
despite receiving the same dose of Na₂EDTA and calcium gluconate (Figs. 3.1-3.2).

**Fractional urinary clearance of calcium and phosphorus**

Induction of hypocalcemia in Experiments 1 and 4 resulted in increased FCa and
FP. FCa increased from 2.9 ± 0.8% (time 0) to 32.3 ± 8% at the end of Na₂EDTA
infusion (120 min), and FP increased from 0.1 ± 0.1% to 7.5 ± 1.3% at the end of the
Na₂EDTA infusion (Fig. 3.7). At the end of the recovery from hypocalcemia, FCa
decreased to 8.9 ± 2.9%, which was greater than the baseline value (P<0.05), and FP
decreased to 1.9 ± 0.2%, which was greater than the baseline value (P<0.05). Induction of
hypercalcemia resulted in an increase of FCa to 59.1 ± 7.3%, while FP continued to
decline to values similar to baseline (0.17 ± 0.1%) (Fig. 3.7). Calcium gluconate
infusions resulted in higher FCa than Na₂EDTA infusions, and Na₂EDTA infusions
resulted in higher FP than calcium gluconate infusions.

In Experiments 2 and 3, during the induction of hypercalcemia, FCa increased
from 3.8 ± 0.4% to 63.0 ± 13.2% (P<0.05) at 120 min, and FP increased from 0.03 ±
0.04% to 0.9 ± 0.6% at 120 min (P<0.05) (Fig. 3.8). During recovery from
hypercalcemia, FCa decreased to 11.4 ± 5.5%, a value statistically higher than the
baseline value (P<0.05), and FP decreased to 0.01 ± 0.01%. Induction of hypocalcemia
resulted in an increase in FCa to 38.0 ± 5.7% at the end of the Na₂EDTA infusion, and an
increase in FP to 5.2 ± 0.9%. Calcium gluconate infusions resulted in higher FCa than
Na₂EDTA infusions, and Na₂EDTA infusions resulted in higher FP than calcium
 gluconate infusions.
In this study we have determined that horses have a higher Ca\(^{2+}\) set-point (1.37 ± 0.05 mmol/L) than human beings and dogs (1.0-1.2 mmol/L) (Felsenfeld and Llach, 1993; Aguilera-Tejero et al., 1996). Serum total and ionized calcium concentrations in horses are greater than those in other domestic animals and humans (Kaneko et al., 1997). The high serum Ca\(^{2+}\) concentration in the horse may be the result of a high Ca\(^{2+}\) set-point. We speculate that horses are not as sensitive as other species to the suppressive effects of Ca\(^{2+}\) on PTH secretion.

There is also evidence that the Ca\(^{2+}\) set-point is dependent upon basal Ca\(^{2+}\) concentrations, and an increase in basal Ca\(^{2+}\) concentrations was associated with an increase in the Ca\(^{2+}\) set-point, suggesting an adaptation of the set-point to existing serum Ca\(^{2+}\) concentrations (Rodriguez et al., 1997; Caravaca et al., 1995; Pahl et al., 1996). Increased extracellular Ca\(^{2+}\) concentrations have been associated with increased intracellular calcium concentrations, and an increase in the Ca\(^{2+}\) set-point (Wallfelt et al., 1988).

The Ca\(^{2+}\) set-point is determined in part by factors that regulate PTH gene expression. Calcitriol (1,25-dihydroxyvitamin D) and extracellular Ca\(^{2+}\) have negative regulatory effects on the PTH gene (Slatopolsky and Delmez, 1996). Calcitriol increases the sensitivity of the parathyroid gland to extracellular Ca\(^{2+}\) by increasing the expression of the CaR and megalin/gp330/LRP-2 mRNA. This results in low PTH production, and shifts the Ca\(^{2+}\)/PTH sigmoidal curve (and the Ca\(^{2+}\) set-point) to the left (down) (Slatopolsky and Delmez, 1996; Brown et al., 1996; Liu et al., 1998). Plasma
concentrations of vitamin D metabolites (1,25-dihydroxyvitamin D, 25-hydroxyvitamin D) in the horse are considerably lower than those in other domestic animals and humans (Mäenpää et al., 1988; Breidenbach et al., 1998). Because horses have low serum calcitriol concentrations, and calcitriol inhibits PTH synthesis and secretion in other species (Slatopolsky and Delmez, 1996), it is possible that higher serum Ca\(^{2+}\) concentrations are required to suppress PTH secretion in the horse, resulting in a higher Ca\(^{2+}\) set-point. A low number of vitamin D receptors (VDR) in equine parathyroid chief cells could reduce the regulatory effect of calcitriol on PTH synthesis and secretion. There is a direct relationship between serum calcitriol concentrations and parathyroid gland VDR protein and mRNA expression in other species (Naveh-Many et al., 1990; Beckerman and Silver, 1999). Parathyroid glands from humans and rats with chronic renal failure and uremia contained 30-50% of the number of VDR found in controls (Korkor et al., 1987; Merke et al., 1987). Based on the vitamin D/VDR relationship in the parathyroid glands in other species, we speculate that horses may have a relatively low number of VDR in their parathyroid glands. The physiology of regulation of VDR in the horse parathyroid gland has not been reported.

Brown et al. (1993) cloned a calcium-sensing receptor (CaR) in parathyroid chief cells that binds Ca\(^{2+}\) and other cations and regulates PTH secretion by sensing changes in phosphoinositide metabolism and cytosolic Ca\(^{2+}\) concentration. Activation of the CaR results in decreased PTH secretion, and a lower Ca\(^{2+}\) set-point, while decreased expression or activity of the CaR results in an increased Ca\(^{2+}\) set-point (Kifor et al., 1996). Humans with uremia have a 50% decrease in the number of CaR in the parathyroid chief cells (Kifor et al. 1996), which would be sufficient to account for the
abnormally increased Ca\(^{2+}\) set-point in these patients. Other proteins such as megalin/gp330/LRP-2, which is an endocytic receptor with calcium-sensing properties in the parathyroid gland and other tissues (Lundgren et al., 1994; Hjälm et al., 1996), may play an important role in the regulation of PTH synthesis and secretion. Decreased mRNA expression and protein synthesis for megalin/gp330/LRP-2 have been demonstrated in parathyroid adenomas and are associated with an increased in Ca\(^{2+}\) set-point in these patients (Farnebo et al., 1998). Whether the high Ca\(^{2+}\) set-point in the horse results from a relatively low expression and/or activity of proteins in calcium-sensing system remains to be determined.

An increased Ca\(^{2+}\) set-point has been reported in association chronic renal failure in humans (Brown et al., 1982), and is likely the cause of the hypercalcemia in affected individuals. The Ca\(^{2+}\) set-point has not yet been studied in sick horses. In our laboratory, we have studied septic horses with low Ca\(^{2+}\) concentrations that do not display an increase in serum PTH concentration of the magnitude expected based on the severity of the hypocalcemia (Toribio et al 2001). We hypothesize that a low Ca\(^{2+}\) set-point in these horses could reduce parathyroid gland synthesis/secretion of PTH. Possible mechanisms for reduction in PTH synthesis/secretion in this setting include increased activity of the calcium-sensing system and/or effects of local and systemic factors including inflammatory mediators (Nielsen et al., 1997; Carlstedt et al., 1999).

Although hysteresis has been reported in humans (Conlin et al., 1989) and dogs (Aguilera-Tejero et al., 1996), this is the first study to report the presence of hysteresis in the Ca\(^{2+}\)/PTH relationship in horses. We found that, at the same serum Ca\(^{2+}\) concentrations, PTH values were higher during induction of than during recovery from
hypocalcemia, and were higher during recovery from than during induction of hypercalcemia. We believe, based on the results of this study, that equine parathyroid glands are able to detect the direction of change in serum Ca\(^{2+}\) concentration during both hypocalcemia and hypercalcemia. Although it is not clear why the parathyroid gland responds differently to changes in calcium concentrations depending upon the direction of concentration change, previous studies have suggested that PTH release is not determined only by the absolute serum Ca\(^{2+}\) concentration, but also by the rate of serum Ca\(^{2+}\) change (Kwan et al., 1993; Brent et al., 1988; Grant et al., 1990; Cunningham et al., 1989). An "anticipatory response" of the parathyroid gland is expected to be advantageous, enabling the parathyroid gland chief cells to respond more rapidly to changes in serum Ca\(^{2+}\) concentrations.

A decrease in the rate of PTH secretion during hypocalcemia (hysteresis) may result from extracellular calcium-dependent intracellular PTH degradation (Kwan et al. 1993, Habener et al. 1984). However, this mechanism has been questioned because alterations in intracellular PTH degradation in response to changes in extracellular calcium concentrations require at least 40 min (Habener et al. 1984) and PTH secretion decreased within minutes of an increase in extracellular Ca\(^{2+}\) concentration in ours and other studies (Conlin et al., 1989; Aguilera-Tejero et al., 1996). Parathyroid fatigue, as a result of rapid depletion of intracellular PTH stores, has been considered a possible cause of hysteresis during hypocalcemia (Kwan et al., 1993). However, in the present study, we used a slow (120 min) induction model of hypocalcemia, and most horses were capable of maintaining increased serum PTH concentrations throughout the experiment. A previous study in dogs, using variable rates of induction of hypocalcemia, found no differences in
PTH secretion during induction and recovery from hypocalcemia, nor in maximal PTH concentrations (Aguilera-Tejero et al., 1996). Likewise, humans had comparable maximal PTH concentrations whether hypocalcemia was induced in 60 or 120 min (Brent et al. 1988). In addition, Aguilera-Tejero et al. (1996) found that hysteresis was a reproducible phenomenon in dogs after two sequential cycles of hypocalcemia indicating that that hysteresis may not be completely explained by PTH depletion.

All horses with induced hypocalcemia (Experiments 1 and 4) reached a serum PTH concentration plateau. Four of these horses had a decline in serum PTH concentration to approximately 80% of PTHmax when serum Ca\(^{2+}\) concentrations declined below 1.1 mmol/L, and before the Na\(_2\)EDTA infusion was discontinued. Parathyroid gland exhaustion or fatigue may have been responsible for the decreased PTH secretion in these horses. It is also possible that this decline in serum PTH may have resulted from impaired PTH synthesis and/or secretion due to other regulatory processes such as alterations in the calcium-sensing system.

Hysteresis is a phenomenon now reported in 3 species. Hysteresis is the response of the parathyroid gland to reductions in the rate of change of the serum Ca\(^{2+}\) concentration or to a reversal in the direction of change in the serum Ca\(^{2+}\) concentration (Lowin et al. 1995; Grant et al., 1990; Aguilera-tejero et al., 1996; Conlin et al., 1989). It may also be a manifestation of parathyroid gland exhaustion during prolonged or severe hypocalcemia (Kwan et al., 1993). Hysteresis may be a mechanism designed to protect against over correction of the serum Ca\(^{2+}\) concentrations. When hypocalcemia is abating, the parathyroid chief cells may decrease PTH secretion to reduce the compensatory calcium-conserving effects of PTH on the kidney and its calcium mobilizing effects on bone (Aguilera-Tejero et al., 1996).
The lowest serum Ca$^{2+}$ concentration in our studies was attained at 110-120 min of Na$_2$EDTA infusion. Based on information from other species, this interval may have been long enough for the parathyroid gland to synthesize new PTH. No information on the time required for equine parathyroid cells to synthesize and secrete PTH is available. Bovine parathyroid cells require approximately 30 min to synthesize and secrete PTH (Habener et al., 1984), and chief cell PTH stores are thought to be sufficient to maintain maximal (hypocalcemia-stimulated) PTH secretion for up to 1.5 h in calves (Mayer and Hurst, 1978). Change in PTH mRNA expression in response to a change in extracellular Ca$^{2+}$ concentrations requires up to 3 h in rats (Yamamoto et al., 1989) and 12 h in cattle (Russell et al., 1983). This response is too slow to support new synthesis of PTH in the short term. However, it is possible that in our horses, a combination of PTH release from cytoplasmic stores, and new synthesis of PTH was sufficient to sustain increased serum PTH concentrations during experimental hypocalcemia.

We, like others (Conlin et al., 1989), found that, during the induction of hypercalcemia, serum PTH concentrations were lower than during the recovery period (Figs. 3.4 and 3.5). It is possible that higher serum PTH concentrations during recovery from hypercalcemia are important to reduce renal loses of calcium. The reason that recovery period serum PTH concentrations remained as low as induction period values when serum Ca$^{2+}$ concentrations were greater than 2.0 mmol/L is unclear. We speculate that in the horse, serum Ca$^{2+}$ concentrations higher than 2.0 mmol/L have no additional stimulatory effect on the calcium-sensing system, and therefore chief cells cannot differentiate directional changes in serum Ca$^{2+}$ concentrations at these high serum Ca$^{2+}$ concentrations. We also speculate that it is advantageous for the parathyroid gland to
sense the absolute serum Ca\(^{2+}\) concentrations, as well as the direction of change in serum Ca\(^{2+}\) concentrations, because this provides better calcium homeostatic control.

In addition to determining that hysteresis was present during both hypocalcemia and hypercalcemia in horses, we found that the order in which hypocalcemia or hypercalcemia is induced affects PTH production by the parathyroid gland. Horses in which hypocalcemia was followed by hypercalcemia (Experiments 1 and 4) secreted more PTH (P<0.05) while hypocalcemic than horses in which hypercalcemia was followed by hypocalcemia (Experiments 2 and 3). Moreover, the sigmoidal relationship between serum Ca\(^{2+}\) and PTH concentrations in horses in which hypocalcemia was induced first was not present when hypocalcemia was preceded by hypercalcemia. It is unclear why horses in Experiments 2 and 3 produced less PTH (for the same dose of Na\(_2\)EDTA and at the same serum Ca\(^{2+}\) concentrations), than horses in Experiments 1 and 4. It is possible that modulation of PTH secretion by the Ca\(^{2+}\)-sensing system may vary according to the extracellular Ca\(^{2+}\) concentrations to which the parathyroid cells are exposed. Although the present study was not designed to sustain (clamp) high serum Ca\(^{2+}\) concentrations, our results were similar to those reported by Sanchez et al. (1996) in which hypercalcemia was sustained in dogs for 90 min. In our study, we increased serum Ca\(^{2+}\) concentrations to almost twice baseline values, which may have been similar to clamping serum Ca\(^{2+}\) at high concentrations. Exposure of chief cells to high extracellular Ca\(^{2+}\) concentrations may have modified Ca\(^{2+}\)-sensing mechanisms such that PTH secretion in response to subsequent hypocalcemia was reduced in both the canine and the equine models.

An increase in serum Ca\(^{2+}\) concentrations resulted in an increase in FCa with minimal changes in FP. We believe that inhibition of serum PTH secretion as a result of
hypercalcemia, as well as activation of the CaR in the distal convoluted tubules of the kidney (Brown, 1999), were the mechanisms responsible for the increased FCa in these horses. Induction of hypocalcemia resulted in an increase in FCa despite high serum concentrations of PTH, and may have reflected excretion of calcium-EDTA chelated complexes into the urine. The increased FP during hypocalcemia probably resulted from PTH-mediated inhibition of phosphate reabsorption in the proximal tubules (Rosol and Capen, 1997).

In conclusion, this study has demonstrated that the Ca\(^{2+}\) set-point in the horse is higher than in other domestic animals and man. We have shown that the Ca\(^{2+}\)/PTH relationship in horses is sigmoidal and displays hysteresis during both hypocalcemia and hypercalcemia, and that extracellular Ca\(^{2+}\) concentrations may affect the response of the parathyroid gland to hypocalcemia. Defining the Ca\(^{2+}\) set-point in horses may help to explain the pathophysiology of hypo and hypercalcemia in this species.


Figure 3.1. Intact PTH and Ca\(^{2+}\) concentrations plotted against time in 12 healthy horses in which hypocalcemia was induced prior to hypercalcemia (Experiments 1 and 4). Na\(_2\)EDTA infusion resulted in increased serum PTH concentrations, while calcium gluconate infusion resulted in decreased serum PTH concentrations. Horses of Experiments 1 and 4 had higher serum PTH and PTH\(_{\text{max}}\) concentrations when compared to horses in which hypercalcemia was induced prior to hypocalcemia (Fig. 3.2). The area under the time concentration curve (AUC) for serum PTH was significantly greater in horses of Experiments 1 and 4 than in horses of Experiments 2 and 3 (Fig. 3.2.). There was no statistical difference in the AUC for Ca\(^{2+}\) concentrations in Experiments 1 and 4 as compared to Experiments 2 and 3 (Fig. 3.2).
Figure 3.2. Intact PTH and Ca\textsuperscript{2+} concentrations plotted against time in 12 healthy horses in which hypercalcemia was induced prior to hypocalcemia (Experiments 2 and 3). The AUC for serum PTH was significantly less in horses during Experiments 2 and 3 as compared to Experiments 1 and 4. There was no significant difference in the AUC for serum Ca\textsuperscript{2+} concentration in Experiments 2 and 3 as compared to Experiments 1 and 4.
Figure 3.3. Intact PTH and \( \text{Ca}^{2+} \) concentrations plotted against time in 4 healthy horses (Experiment 5, control). The horses were infused with 0.9% NaCl, and had no significant changes in serum PTH and \( \text{Ca}^{2+} \) concentrations occurred during the experiment.
Figure 3.4. Intact PTH concentrations (% of PTH_max) plotted against serum Ca^{2+} concentrations in healthy horses (Experiments 1 and 4). Induction of hypocalcemia (1) resulted in a Ca^{2+}/PTH sigmoidal curve (closed squares). During the recovery period (2) PTH concentrations were lower (hysteresis), and the PTH/Ca^{2+} relationship displayed linearity (open squares). Once serum Ca^{2+} reached the reference range (1.55-1.67 mmol/L) hypercalcemia was induced (3) (closed circles). During the recovery period from hypercalcemia (4) PTH concentrations were greater (open circles) than during induction of hypercalcemia (hysteresis).
Figure 3.5. Intact PTH concentrations (as a % of mean PTH\text{max} from Experiments 1 and 4) plotted against serum Ca^{2+} concentrations in healthy horses (Experiments 2 and 3). The induction (1; closed circles) and recovery (2; open circles) from hypercalcemia displayed hysteresis. The induction of hypocalcemia (3; closed squares) was not as sigmoidal and serum PTH concentrations were not as high as in Experiments 1 and 4, but there was hysteresis (4; open squares).
Figure 3.6. Relationship between serum Ca²⁺ and PTH concentrations in 12 healthy horses. PTH concentrations were expressed as a percentage of PTHmax for each horse. The sigmoidal relationship between serum Ca²⁺ and PTH concentrations was calculated using the four-parameter logistic model (dotted line) and the E_max sigmoidal model (Hill’s equation; solid line). The Ca²⁺ set-point was calculated as 50% of PTHmax for each horse. Both regression lines overlapped indicating that alternative mathematical methods to calculate the relationship between serum Ca²⁺ and PTH concentrations can be applied.
Figure 3.7. Induction of hypocalcemia (Experiments 1 and 4) resulted in an increase of FCa from $2.9 \pm 0.8\%$ (time 0) to $32.3 \pm 8\%$ at the end of Na$_2$EDTA infusion (120 min), and FP increased from $0.1 \pm 0.1\%$ to $7.5 \pm 1.3\%$ at the end of the Na$_2$EDTA infusion. At the end of the recovery period from hypocalcemia FCa decreased to $8.9 \pm 2.9\%$, and FP decreased to $1.9 \pm 0.2\%$. Induction of hypercalcemia resulted in an increase of FCa to $59.1 \pm 7.3\%$, while FP continued to decline to values similar to baseline ($0.17 \pm 0.1\%$).
Figure 3.8. During the induction of hypercalcemia (Experiments 2 and 3) FCa increased from 3.8 ± 0.4% to 63.0 ± 13.2% (P<0.05) at 120 min, and FP increased from 0.03 ± 0.04% to 0.9 ± 0.6% at 120 min. During the recovery from hypercalcemia FCa decreased to 11.4 ± 5.5%, which is higher than baseline values (P<0.05), and FP decreased to 0.01 ± 0.01%. During hypocalcemia FCa increased to 38.0 ± 5.7% at the end of the Na<sub>2</sub>EDTA infusion, and FP increased to 5.2 ± 0.9%.
CHAPTER 4

EQUINE PARATHYROID CHIEF CELLS IN VITRO: SECRETION OF PARATHYROID HORMONE (PTH), PTH AND CALCIUM-SENSING RECEPTOR mRNA EXPRESSION, AND EFFECTS OF INTERLEUKIN-1

Abstract

Parathyroid hormone (PTH) is secreted by the chief cells of the parathyroid gland in response to changes in extracellular ionized calcium (Ca\(^{2+}\)) concentrations. In vitro studies on parathyroid cells from different species have improved our understanding of the physiology and pathophysiology of the parathyroid gland. Several conditions in the horses are associated with abnormal parathyroid gland function. In a previous study we found that hypocalcemia and abnormal parathyroid gland function were present in some horses with sepsis. In the present study we evaluated equine parathyroid cell morphology and proliferation, PTH secretion, PTH mRNA and calcium-sensing receptor (CaR) mRNA expression. We also evaluated the effect of interleukin-1β (IL-1β), a cytokine known to be increased during sepsis in humans and animals, on PTH secretion, PTH and CaR mRNA expression. There was an inverse sigmoidal relationship between PTH secretion and Ca\(^{2+}\) concentrations on day 0. PTH secretion in a low Ca\(^{2+}\) (0.8 mM) medium decreased from 100% (day 0) to 13% (day 30). The inhibitory effect of high
Ca\textsuperscript{2+} concentrations on PTH secretion also declined. By day 5 of culture, higher Ca\textsuperscript{2+} concentrations were required to inhibit PTH secretion, and there was a rightward shift (increase) of the Ca\textsuperscript{2+} set-point. After day 10 of culture, there was no significant difference in PTH secretion between low (0.8 mM) and high (2.0 mM) Ca\textsuperscript{2+} concentrations. Parathyroid cells exposed to high Ca\textsuperscript{2+} concentrations had lower PTH mRNA expression (P<0.05) than cells exposed to low Ca\textsuperscript{2+} concentrations. PTH mRNA expression declined from 100% (day 0) to 43% (day 5), and to 25% (day 30). CaR mRNA also decreased from 100% (day 0) to 31% (day 5), and to 16% (day 30). Low and high Ca\textsuperscript{2+} concentrations had no effect on CaR mRNA expression. IL-1\textbeta (2000 pg/ml) decreased both PTH secretion (75%) and PTH mRNA expression (73%) (P<0.05). IL-1\textbeta concentrations greater than 200 pg/ml resulted in significant (P<0.05) overexpression of CaR mRNA (up to 142%). The effects of IL-1\textbeta were blocked by an IL-1 receptor antagonist (IL-1ra). We conclude that the decreased responsiveness of parathyroid cells to Ca\textsuperscript{2+} from 0 to 30 days could be explained in part by the reduced CaR expression. We also believe that IL-1 plays an important role in regulating PTH secretion in the horse.

Introduction

Parathyroid hormone (PTH) is secreted by the chief cells of the parathyroid gland, and plays an important role in calcium homeostasis. Parathyroid hormone secretion is altered by small physiological changes in extracellular ionized calcium (Ca\textsuperscript{2+}) concentrations, and there is an inverse sigmoidal relationship between serum Ca\textsuperscript{2+} concentrations and PTH secretion (Brown, 1983; Mayer and Hurst, 1978; Brent et al.,
1988; Aguilera-Tejero et al., 1996; Chapter 3), represented by a four-parameter model (Brown, 83), 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 LDL-receptor superfamily) (Brown et al., 1993; Lundgren et al., 1994; Hjâlm 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; Liu et al., 2001; Sakaguchi et al., 1987; Fasciotto et al., 1989). 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 to other species (Toribio et al., 2001), high urinary fractional clearance of calcium (Toribio et al., 2001), low serum concentrations of vitamin D (1,25-dihydroxyvitamin D, 25-hydroxyvitamin D) (Mäenpää et al., 1988; Breidenbach et al., 1998), and an increased Ca\(^{2+}\) set-point (Chapter 3).

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), pseudohyperparathyroidism (Marr et al., 1989), hypoparathyroidism (Couëtil et al., 1998), vitamin D toxicity (Harrington and 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 and IL-6 (Seethanathan et al., 1990; Barton et al., 1998; Bueno et al., 1999) have been shown in other species to increase the expression of the CaR mRNA in parathyroid cells and decrease PTH secretion (Nielsen et al., 1997; Carlstedt et al. 1999). Because sepsis in horses is often associated with increased concentrations of IL-1 and IL-6, we believe that these inflammatory mediators may directly or indirectly suppress PTH secretion.

The objectives of this study were, 1) to evaluate equine parathyroid cell morphology and proliferation \textit{in vitro}, 2) to determine the functionality (measured as PTH secretion) of chief cells to changes in extracellular Ca\(^{2+}\) concentrations, 3) to measure PTH secretion, PTH mRNA and CaR mRNA expression in equine chief cells for 30 days, and 4) to assess the effect of interleukin-1 on PTH secretion, PTH mRNA and CaR mRNA expression.
Preparation of Parathyroid Cells

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 phosphate buffered saline (PBS) containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (20 μg/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 CaCl₂ (1.0 mM), MgCl₂ (0.5 mM), and 0.5% BSA. Dissociated cells were obtained by collagenase P (2 mg/ml, Roche Molecular Biochemicals, Indianapolis, IN, USA) and Dnase I (50 μg/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 μg/ml), gentamicin (4 μg/ml), CaCl₂ (1.0 mM), MgCl₂ (0.5 mM), and 0.5% BSA, in a 5% CO₂ incubator (Forma Scientific Inc, Marietta, OH, USA) for 45-60 min at 37°C, with continuous shaking (100 rpm), 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 two times by centrifugation at 100g for 5 min to reduce cellular debris and fibroblast contamination. Cell viability after processing, assessed by trypan blue dye exclusion was estimated 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 μg/ml), gentamicin (4 μg/ml), ITS (insulin, transferrin, selenous acid, BSA, and linoleic acid), 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 using 5-10% FBS and from a report (Racke and 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. We used low concentrations of streptomycin and gentamicin (polycations) to reduce intracellular Ca\(^{2+}\) mobilization, which inhibits PTH secretion (Katz et al., 1992).

Morphological studies

For morphological studies, the parathyroid cells were cultured in parathyroid medium containing a Ca\(^{2+}\) concentration of 0.8 mM and 5% FBS to increase cell attachment. Phase contrast light microscopy (40X, 100X, and 200X) was used to examine equine parathyroid chief cell morphology and growth.

The proliferative life span of parathyroid cells was determined by subculturing the cells every 5 days as previously described by Brandi et al. (1986). The cells were seeded at a density of 1x10^4 cells per well in 35mm multiwell plates. Twenty-four, 48, 72, and 96 (day 30) h after seeding, the cells were detached with trypsin/0.5% EDTA and counted in triplicates using a hemocytometer.
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 chief cells in suspension. The cells (1x10^4 cells) were incubated in triplicate at 37 °C, 5% CO\(_2\), for 3 h in parathyroid culture medium (Ca\(^{2+}\)=0.5-3.0 mM) on a shaker at 100 rpm. At the end of the incubation time and after centrifugation at 500g for 5 min, the medium was aspirated and frozen at -20 °C until analysis for intact PTH (1-84), 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 CaRmRNA expression**

The effect of low Ca\(^{2+}\) concentration on PTH secretion was determined in triplicate every 2-3 days for 30 days. Parathyroid chief cells (1x10^4) were incubated in parathyroid culture medium with a Ca\(^{2+}\) concentration of 0.8 mM for 3 h. At the end of the incubation time, and after centrifugation at 500g 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 varying Ca\(^{2+}\) concentrations (0.8-2.0 mM) on PTH secretion was determined in triplicate wells of parathyroid chief cells (1x10^4 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 (Ca\(^{2+} = 0.8\) mM) for each day.

The effect of low (0.8 mM) and high (2.0 mM) \(Ca^{2+}\) concentrations on CaR and PTH mRNA expression was evaluated in duplicate using 0.5x10\(^6\) chief cells on days 1 and 2.

The expression of PTH and CaR mRNA were determined in duplicate using 0.5x10\(^6\) parathyroid chief cells (Ca\(^{2+} = 0.8\) mM) by Northern blot analysis on days 0, 1, 2, 5, 10, 15, 20, 25, 30, and expressed as PTH mRNA/GAPDH mRNA or CaR mRNA/GAPDH mRNA ratio. Controls consisted of PTH mRNA or CaR mRNA/GAPDH mRNA expression on day 0 (100% expression).

The effect of IL-1\(\beta\) on PTH secretion, and PTH and CaR mRNA expression

The effect of human recombinant IL-1\(\beta\) (R&D systems Inc., Minneapolis, MN, USA) (20, 200, and 2000 pg/ml) on PTH secretion, and PTH and CaR mRNA expression was examined in parathyroid chief cells (0.5x10\(^6\)) in duplicate during incubation for 24 h in low \(Ca^{2+}\) (0.8 mM) medium. The concentrations of IL-1\(\beta\) used in this study were chosen from previous reports based on the effect of IL-1\(\beta\) on bovine parathyroid cell function (Nielsen et al., 1997).

To determine if the effect of IL-1\(\beta\) on PTH secretion, and PTH and CaR mRNA expression was mediated through an IL-1-specific receptor, a human recombinant IL-1 receptor antagonist (IL-1ra, Amgen Inc., Thousand Oaks, CA, USA) at 100 ng/ml was added to the medium (Ca\(^{2+} = 0.8\) mM) 2 h previous to the addition of IL-1\(\beta\) (2000 pg/ml).
The cells were incubated for 24 h after adding IL-1β. In addition, parathyroid chief cells were also incubated with IL-1ra alone (100 ng/ml).

**PTH, CaR, and GAPDH mRNA expression**

To measure PTH mRNA expression, a 399-base pair probe was generated by polymerase chain amplification of canine PTH cDNA (Rosol et al., 1995) (primers: 5'-GTGTGTGAAGATGATGTCTGC; 5'-TTGTTGCCCTATGCTGTCTA). For CaR mRNA expression, a 1.6 kb CaR cDNA was generated by reverse transcription polymerase chain reaction (primers: 5'-TGGAACTGGGTGGGCCACAAAT, 5'-CCAGCAGGAACTGCAAGGTTGAG) of equine parathyroid gland total RNA, subcloned into pCR4-TOPO (Invitrogen, Carlsbad, CA, USA), sequenced, and used as a probe. For GAPDH mRNA expression, a 780-bp fragment of human GAPDH cDNA was used.

The probes were labeled with [³²P]-dATP (NEN life Science Products, Boston, MA, USA) using a random primers DNA labeling system (Life Technologies, Grand Island, New York, USA) to 1 x 10⁹ cpm/µg of DNA.

Total RNA was extracted from 0.5x10⁶ parathyroid chief cells using guanidine thiocyanate and silica-based filters (RNAqueous-4PCR kit, Ambion, Austin, TX, USA). Total RNA (10µg) was electrophoresed on denaturing formaldehyde (6%) agarose (1%) gels. After electrophoresis for 3 h at 70 volts, the RNA was transferred onto nylon membranes (Stratagene, La Joya, CA, USA). The membranes were UV irradiated with a Stratalinker UV Crosslinker 1800 (Stratagene), prehybridized in Ultrahyb solution (Ambion) for 1 h at 42 °C and hybridized in Ultrahyb solution with denatured herring
sperm DNA and cDNA probe (1 x 10^6 cpm/ml of hybridization solution) for 16 h. After hybridization the membranes were washed twice for 5 min in 2X SCC/0.1% SDS at room temperature and twice with 0.1X 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 0.1X 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 (1-84) was measured with a two site immunochemiluminoimetric 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; Chapter 2). PTH concentrations were normalized to cell protein content.

Protein determination

Cell protein was extracted from the parathyroid cells using RIPA cell lysis buffer (PBS, 1% NP40, 0.1% SDS, 1% deoxycholate, 2mM EDTA). Protein concentrations were measured using the Coomassie brilliant blue binding protein assay (Bradford, 1976) using bovine serum albumin as the protein standard.
Electrolytes measurements

Electrolytes (Ca$^{2+}$, Mg$^{2+}$, 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 mean ± SD. Comparisons between groups were made using the t-test or the Mann-Whitney rank test. Comparisons among groups were made using one-way ANOVA with Tukey's test for multiple comparisons, or the Kruskal-Wallis one-way ANOVA with Dunn's test for multiple comparisons depending on the data distribution (Fisher and 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. Statistical analyses were performed with SigmaStat 2.01 for Windows (SPSS Inc, Chicago, IL, USA). P values less than 0.05 were considered statistically significant.

Results

Morphological studies

Parathyroid cells in suspension varied from single cells to clumps of cells (Fig. 4.1a). When 5% of FBS was added to DMEM/F12 medium (Ca$^{2+} = 0.8$ mM), attachment
of the parathyroid chief cells to culture dishes was evident at 6 h of incubation, and by day 2 of culture approximately 90% of the cells were attached to the plates (Fig. 4.1b,c). The cells were polygonal, grew in colonies and spread out to from a monolayer. The number of spindle-shaped cells on day 2, presumambly fibroblasts, was minimal (<1%) (Fig. 4.1c).

Parathyroid cells were harvested every 5 days for 30 days to measure doubling times. On day 5 the doubling time was estimated to be 38 ± 8 h, on day 10 it was 48 ± 10 h, and on day 30 it was 68 ± 15 h (Fig. 4.2).

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. 4.3). PTH secretion ranged from 455 ± 102 ng/mg of cell protein (Ca\(^{2+}\) = 0.8 mM, 100% PTH secretion) to 55 ± 27 ng/mg of cell protein (Ca\(^{2+}\) = 3.0 mM).

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. 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\(_{\text{max}}\) was 1.30 ± 0.05 mM (Fig. 4.3), which is similar to the Ca\(^{2+}\) set point that we measured in healthy horses (1.37 ± 0.05 mM) (Chapter 3).
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 of cell protein (100%). By day 5 of culture PTH secretion declined to 68 ± 12%, by day 10 to 33 ± 6%, and by day 30 to 13 ± 3% to that of day 0 (Fig. 4.2). The inhibitoy 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, there was a rightward shift (increase) of the Ca\(^{2+}\) set-point (Fig. 4.4) because higher Ca\(^{2+}\) concentrations were required to inhibit PTH secretion. After day 10 of culture there was no significant difference in PTH secretion between low and high Ca\(^{2+}\)concentrations (Fig. 4.4).

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. 4.5). There was a decline in CaR mRNA expression that was time-dependent, but was not statistically associated with Ca\(^{2+}\) concentrations. PTH mRNA expressions 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. 4.5).

CaR and PTH mRNA expression declined over time when compared to day 0. By day 2 of culture, CaR mRNA expression declined to 41 ± 7%, by day 5 to 31 ± 6%, by day 10 to 21 ± 7%, and by day 30 to 16 ± 6% of controls (day 0)(Fig. 4.6). PTH mRNA expression declined, but not as rapidly as CaR mRNA. By day 2, PTH mRNA expression
was 79 ± 5%, by day 5 it was 43 ± 13%, by day 10 it was 35 ± 11%, and by day 30 it was 25 ± 8% of controls (day 0) (Fig. 4.6).

The effect of IL-1β on PTH secretion, and PTH and CaR mRNA expression

When parathyroid cells were incubated with increasing concentrations of human recombinant IL-1β, only an IL-1β concentration of 2000 pg/ml decreased both PTH secretion (75%) and PTH mRNA expression (73%) (P<0.05) (Fig. 4.7). No statistically significant difference in PTH secretion and PTH mRNA expression at lower IL-1β concentrations was detected when compared to controls; however, a trend for reduced PTH secretion and mRNA expression was present at 200 pg/ml of IL-1β (Fig. 4.7). In contrast, IL-1β concentrations of ≥ 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-1β (2000 pg/ml), there was no significant difference in PTH secretion, and PTH and CaR mRNA expression when compared to controls (no IL-1β), suggesting that the effects of IL-1β 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 to controls.
In this study we demonstrated that equine parathyroid chief cells cultured in vitro were responsive to changes in extracellular Ca$^{2+}$ concentrations for up to 5 days after collection. We also found that PTH secretion in adherent parathyroid chief cells when compared to cells in suspension (day 0) was significantly lower, and the Ca$^{2+}$ set-point was shifted rightwards on day 5, due to decreased responsiveness to high Ca$^{2+}$ concentrations. These findings suggest that equine parathyroid chief cells must be used early after collection when evaluating their responsiveness to changes in extracellular Ca$^{2+}$ concentrations. Similar findings were reported with bovine parathyroid cells, in which there was decreased parathyroid cell responsiveness (PTH secretion) to changes in Ca$^{2+}$ 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 Ca$^{2+}$ 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, in our study, despite the low expression of CaR mRNA (day 5), the cells were still responsive to changes in extracellular Ca$^{2+}$ 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 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; Hjälm et al., 1996). Decreased mRNA expression and protein synthesis for megalin/gp330/LRP-2 may contribute to determining the Ca^{2+} 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 by 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 to PTH mRNA expression. In addition to decreased CaR mRNA expression, there was a rightward shift of the Ca^{2+} set-point, which we believe resulted from the decreased expression of CaR mRNA, as previously reported (Kifor et al., 1996). It is unclear why there was a decrease in CaR mRNA expression in equine chief cells, but we believe 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 Ca^{2+} 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 Ca^{2+} 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 to 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 like megalin/gp330/LRP-2 (Fujii et al., 1991; Fujimi et al., 1991; Lundgren et al., 1994; Kilav et al., 1995; Slatopolsky and Delmez, 1996; Hjälm et al., 1996; Fasciotto et al., 2000).

Interleukin-1 is a cytokine with neuroendocrine, endocrine, metabolic, cardiovascular, and proinflammatory functions (Dinarello, 2000). Our study showed that IL-1\(\beta\) 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\(\beta\) to inhibit PTH secretion and increase CaR expression, was present in parathyroid tissue slices but not in dispersed parathyroid cells. The effect of IL-1\(\beta\) 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\(\beta\). 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.
Several conditions in the horse are associated with abnormal calcium homeostasis (Harrington and Page, 1983; Elfers et al., 1986; Marr et al., 1989; Ronen et al., 1992; Dart et al., 1992; Beyer et al., 1997; Frank et al., 1998; Couëtil et al., 1998; Toribio et al., 2001; Aguilera-Tejero et al., 2001). However, there is limited information on equine parathyroid gland function. We evaluated equine parathyroid cell proliferation, PTH secretion, PTH and CaR mRNA expression, as well as the effect of interleukin-1β on PTH secretion, PTH and CaR mRNA expression.

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 in vitro. 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-1β, an inflammatory mediator known to be increased in septic horses, inhibited PTH secretion and mRNA expression, and increased CaR mRNA expression. We consider that overexpression of CaR from the IL-1β treatment could have increased Ca^{2+} sensitivity in the equine parathyroid chief cells, resulting in decreased PTH secretion. These findings indicate that IL-1β may play a role in regulation of equine parathyroid gland function in vivo. The 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.


Figure 4.1. Phase contrast microscopy of parathyroid chief cells. Cells in suspension varied from single cells to clumps of cells (a, 40X). The cells were polygonal, growing in colonies and spreading out to form a monolayer (days 3-5) (b,c, 100X). The number of spindle-shaped cells on day 2, presumably fibroblasts (arrow), was minimal (c).
Figure 4.2. PTH secretion of equine parathyroid chief cells cultured in a low Ca\(^{2+}\) (0.8 mM) medium. PTH secretion decreased over time. On day 5 of culture, PTH secretion was 68%, on day 10 was 33%, and after day 13, PTH secretion was below 18% compared to day 0. To determine the doubling times, chief cells were harvested every 5 days for 30 days. Doubling time increased from 28 h on day 2 to 68 h on day 30 (insert graph).
Figure 4.3. Equine parathyroid chief cells on day 0 (collection day) were exposed to different Ca\(^{2+}\) concentrations for 3 h. 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\(_{\text{max}}\) was calculated to be 1.3 mM.
Figure 4.4. Reduction in responsiveness of equine parathyroid chief cells to changes in \( \text{Ca}^{2+} \) concentrations. By day 5 of culture chief cells remained responsive to changes in \( \text{Ca}^{2+} \); however, there was a rightward shift in the \( \text{Ca}^{2+} \) set-point from 1.38 mM (day 0) to 1.57 mM (dotted line) because higher \( \text{Ca}^{2+} \) concentrations were required to inhibit PTH secretion. After day 10 of culture there was no significant difference in PTH secretion between low and high \( \text{Ca}^{2+} \) concentrations. Control (100% PTH secretion) for each day was the PTH secreted at a \( \text{Ca}^{2+} \) concentration of 0.8 mM.
Figure 4.5. Effect of low (0.8 mM) and high (2.0 mM) Ca\(^{2+}\) concentrations on CaR mRNA and PTH mRNA expression. A significant effect of low or high Ca\(^{2+}\) concentrations on CaR mRNA expression was not detected on either day 1 or 2. The decline of CaR mRNA was time dependent and not statistically associated to 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 chief cells exposed to high Ca\(^{2+}\) concentrations had lower PTH mRNA expression than cells exposed to low Ca\(^{2+}\) concentrations *(P<0.05).*
Figure 4.6. PTH and CaR mRNA expression, and PTH secretion in parathyroid chief cells cultured for 30 days in a low Ca^{2+} medium. PTH secretion, PTH and CaR mRNA expression decreased over time when compared to 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 to 25% of the controls (day 0).
Figure 4.7. Equine parathyroid chief cells incubated with human recombinant IL-1β (0-2000 pg/mL) and human recombinant IL-1 receptor antagonist (IL-1ra) (100 ng/ml). Parathyroid cells incubated with IL-1β had a significant (P<0.05) decrease in both PTH secretion (75%) and PTH mRNA expression (73%) (P<0.05) when IL-1β concentration was 2000 pg/ml. No significant difference in PTH secretion and PTH mRNA expression was detected at lower IL-1β concentrations; however, a trend for low PTH secretion and PTH mRNA expression was present. IL-1β concentrations greater than 200 pg/ml resulted in a significant (P<0.05) overexpression of CaR mRNA (up to 142%). The effects of IL-1β on PTH secretion, and PTH mRNA and CaR mRNA expression were blocked when an IL-1ra was added to the culture medium prior to IL-1β. No effect of IL-1ra on PTH secretion, and PTH and CaR mRNA was detected when compared to controls.
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CHAPTER 5

MOLECULAR CLONING AND EXPRESSION OF EQUINE CALCITONIN, CALCITONIN GENE-RELATED PEPTIDE-I, AND CALCITONIN GENE-RELATED PEPTIDE-II

Abstract

In this study we describe the cloning of equine calcitonin (CT), calcitonin-gene related peptide (CGRP)-I, and CGRP-II cDNA. We also describe a divergent form of CGRP (CGRP-I). The nucleotide sequence for equine preproCT (835 bp) consisted of a 102 bp 5' UTR, a 423 bp coding region for a 140-amino acid protein, and a 310 bp 3' UTR. Equine calcitonin has greatest homology (>85%) to human, rat and mouse subgroups of calcitons. Equine preproCGRP-I (702 bp), consisted of a 102 bp 5' UTR, a 384 bp coding region for a 127-amino acid protein, and a 216 bp 3' UTR. Equine CGRP-I (37aa) has low homology (<59%) to CGRPs of other species. The signal and terminal peptides for equine calcitonin and CGRP-I were identical, indicating that these peptides are encoded by a gene equivalent to the human CALCI gene. Equine preproCGRP-II (960 bp), consisted of a 69 bp 5' UTR, a 390 bp coding region for a 129-amino acid protein, and a 501 bp 3' UTR. Equine CGRP-II (37 aa) has >80% homology to chicken, human, rat, ovine, swine, and bovine CGRPs. The homology between equine
CGRP-I and CGRP-II is low (56%). The high homology of equine CGRP-II, and the low homology of equine CGRP-I to CGRP in other species were unexpected findings, because the homology of all known CGRP peptides was reported to be high in mammals. Northern blot analysis of equine tissues revealed that expression of calcitonin mRNA was restricted to the thyroid gland, while expression of CGRP-I and CGRP-II mRNA was present in several regions of the nervous system and other tissues.

**Introduction**

Studies on calcitonin gene expression have revealed alternative splicing of the primary transcript (Rosenfeld et al., 1981; Amara et al., 1985; Nelkin et al., 1988; Cumaraswamy et al., 1993). Calcitonin (CT), a calcium-lowering hormone synthesized by the C-cells (parafollicular cells) of the thyroid gland (Copp et al., 1962), is a single-chain peptide of 32 aa residues whose amino-terminus contains a cyclic structure involving seven amino acids bridged by a disulfide bond (Becker et al., 1996). The human polypeptide precursor of calcitonin, preprocalcitonin (preproCT), contains 141 amino acids (Le Moullec et al., 1984). Human procalcitonin (PCT) contains 116 amino acid residues, and consists of a centrally positioned immature CT that contains 33 amino acids, a 57-amino acid N-terminal peptide, and a 21-amino acid carboxyl terminal peptide (CCP or katacalcin) (Becker et al., 1996). In 1981, Rosenfeld et al. demonstrated that the calcitonin gene was the source of an alternative mRNA, and the corresponding peptide was named calcitonin gene-related peptide (CGRP).
Human CGRP-I (or CGRP-α) is a 37 amino acid peptide which differs from human CGRP-II (or CGRP-β) by three amino acids (Amara et al., 1985; Steenbergh et al., 1985). In humans, both CT and CGRP-I are encoded by the CALC-I gene (Becker et al., 1996). CGRP-II originates from the CALC-II gene, for which there is no known alternative splicing of the primary transcript (Steenbergh et al., 1986; Becker et al., 1996). Alevizaki et al. (1986), have identified a region within the CALC-II gene which has the potential to encode a novel calcitonin-like peptide. However, no corresponding mRNA has been identified, and it is unclear whether this sequence can be expressed. CGRPs are pleiotropic neuropeptides found in the central and peripheral nervous system (Brain and Cambridge, 1996; Van Rossum et al., 1997), and also are present in C-cells of the thyroid gland (Sabate et al., 1985; Becker et al., 1996; Van Rossum et al., 1997). CGRPs have been implicated in a diverse array of physiological processes including neurotransmitter activity, vasodilation, relaxation of smooth muscle, cardiac acceleration, synthesis and function of nicotinic acetylcholine receptor, nociception, carbohydrate metabolism, gastrointestinal motility, neurogenic inflammation, gastric acid secretion, inhibition of bone resorption, and anti-inflammatory activity by reducing TNF-α concentrations (Goltzman and Mitchell, 1985; Brain and Cambridge, 1996; Valentijn et al., 1997; Feng et al., 1997; Monneret et al., 2000; Gangula et al., 2000).

Hypocalcemia is a common finding in human patients with sepsis, and serum calcitonin, procalcitonin, and CGRP concentrations have been reported to be increased in many of these cases (Chesney et al., 1983; Sperber et al., 1990; Parida et al., 1998; Lind et al., 2000). Similarly, hypocalcemia is common in horses with sepsis and endotoxemia (Toribio et al., 2001). The importance of peptides of the calcitonin gene family in the
pathogenesis of hypocalcemia in septic human patients and horses is unknown. Because there were no published studies on the equine calcitonin gene family, we cloned and sequenced the cDNA encoding for equine calcitonin, CGRP-I and CGRP-II. Investigations of CT, PCT, and CGRP in horses with enterocolitis and endotoxemia will provide useful information on the pathogenesis of hypocalcemia associated with sepsis and endotoxemia in animals and man.

Materials and Methods

cDNA synthesis and library construction

Total RNA was extracted from equine thyroid tissue with Trizol (Gibco BRL, Grand Island, NY, USA). Poly (A)^+ mRNA was isolated using oligo(dT)-cellulose columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA). First strand cDNA synthesis was performed using the Great Lengths cDNA synthesis kit (Clontech, Palo Alto, CA, USA). The cDNA was then cloned into λTriplEx (Clontech, Palo Alto, CA, USA) and packaged with the Gigapack III Gold Packaging Extract (Stratagene, La Joya, CA, USA). The directionally-cloned equine thyroid cDNA library contained 1.2 x 10^6 recombinants, and the cDNA insert size ranged from 0.5-4.0 kb.
Luria-Bertani/agar/MgSO₄ top agarose dishes (150mm) were plated with approximately 5 x 10⁴ pfu per dish, using E. coli XL-1 blue as the phage host. The plates were incubated at 42°C for 4 h and at room temperature overnight. When plaques were confluent the plates were stored at 4°C. Nylon membranes (Osmonics, Minnetonka, MN, USA) were placed on the top agarose for 2-3 min. Subsequently the cDNA was denatured in 1.5M NaCl/0.5M NaOH, neutralized in 1.5M NaCl/0.5M Tris-HCl pH 8.0, and rinsed in 0.2M Tris-HCl pH 7.5, 2X SSC. The nylon membranes were dried, and the DNA was bound to the membranes by UV crosslinking in a Stratalinker UV Crosslinker 1800 (Stratagene, La Joya, CA, USA). A 301 bp probe was generated by PCR of ovine calcitonin cDNA (Cumaraswamy et al., 1993). Primers (5'-CTCGACCTCTCCTGAAAATC, 5'-CTCCAGAGCTAAGCGGTGCA) were designed to amplify exons 1-3, and the amplified product hybridized to common regions of CT and CGRP. The probe was radiolabeled with (α-32P)dATP (NEN Life Science Products, Boston, MA, USA) using a DNA-labeling kit (Gibco BRL, Grand Island, NY, USA) to 1.5 x 10⁹ cpm/µg of DNA. The nylon membranes were prehybridized in 50% formamide/5X SSPE/5X Denhardt's solution/0.1% SDS/100 µg/mL denatured herring sperm DNA for 4 h at 42 °C. The prehybridization solution was removed, and new solution and probe (5 x 10⁶ cpm/nylon membrane) were added. The nylon membranes were incubated at 42°C for 20 h. The membranes were washed once in 2X SSC/0.5% SDS for 20 min at room temperature, and twice in 1X SSC/0.1% SDS at 60 °C for 15 min, and then were dried and wrapped in plastic film. Initial screening to detect positive
clones was performed using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA, USA). When positive clones were detected, the membranes were exposed to autoradiography film (Eastman Kodak Company, Rochester, NY, USA) at -80 °C. Positive clones were screened two additional times to obtain single clones. The phage DNA was isolated with the Wizard Plus Lambda DNA purification system (Promega Corporation, Madison, WI, USA). Plasmids (pTriplEx) were generated by cre recombinase-mediated site-specific recombination in E. coli BM25.8 (Clontech, Palo Alto, CA, USA). The plasmid was purified with Wizard Plus Minipreps DNA purification system (Promega Corporation, Madison, WI, USA).

DNA sequence analysis

Approximately 1 x 10^6 clones were screened. Four clones hybridized to the CT/CGRP probe. The phage and plasmids inserts were sequenced with λ TriplEx-specific sequencing primers (Clontech, Palo Alto, CA, USA), the T7 promoter primer, and equine CT and CGRP-specific primers, using dideoxy nucleoside triphosphates and an ABI PRISM 373XL DNA sequencer (PE Biosystems, Foster City, CA, USA). The nucleotide and protein sequences were analyzed and compared to other species using Align Plus software (Sci-ed Software, Durham, NC, USA).
Northern blot analysis

Total RNA (20μg) from 20 different equine tissues was electrophoresed on denaturing formaldehyde (6%) agarose (1%) gels. After electrophoresis for 3h at 65 volts, the RNA was transferred onto nylon membranes (Stratagene, La Joya, CA, USA). The membranes were UV irradiated (Stratagene, La Joya, CA, USA), prehybridized in QuikHyb solution (Stratagene, La Joya, CA, USA) for 20 min at 68°C and hybridized in QuikHyb solution with denatured herring sperm DNA and the cDNA probe (1.25 x 10^6 cpm/ml of hybridization solution) for 1 h. For equine calcitonin a 298 bp probe was generated by PCR of exon 4 (primers, 5'-CTGGGCACATACACGCAGGA; 5'-TGCTCTCTCTCTCCATCTG). For equine CGRP-I (clone B1) a 224 bp probe was generated by PCR of exon 5 (primers, 5'-TGCTGGGAGTATGGCGAACA; 5'-GAGTGATTTCAATGTCCAGAAGCAT). For equine CGRP-II (clone A1), a 318 bp probe was generated by PCR of exon 6 (primers, 5'-TGACAACAGCTTGGTGAAGG; 5'-AGGCATAGCATCACCAGTAGCA). There was no homology between the CGRP-II probe and CGRP-I. Initial screening of the membranes was performed using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA, USA). When hybridization bands were detected, the membranes were exposed to autoradiography film (Eastman Kodak Company, Rochester, NY, USA) at -80 °C.
Results

Cloning of equine calcitonin and CGRPs

Screening of approximately 1 x 10^6 clones revealed 4 clones (A1, A4, B1, and B2) that hybridized with the CT/CGRP-specific probe, and contained complete cDNA sequences for calcitonin and CGRP. Sequence analysis revealed 3 different cDNA products, with a length of 835 bp for calcitonin (A4), 702 bp for CGRP-I (B1, B2), and 960 bp for CGRP-II (A1). Clone B2 sequence was 100% identical to clone B1, therefore only clone B1 was analyzed.

Nucleotide and deduced amino acid sequences were compared to sequences available in the NCBI GenBank (http://www.ncbi.nlm.nih.gov)

Equine calcitonin sequence analysis

Clone A4 (835 bp), contained the cDNA sequence for equine preprocalcitonin (Fig. 5.1). The preprocalcitonin cDNA consisted of 102-bp of the 5' UTR, the 423-bp coding region, and 310-bp of the 3' UTR, including a 19 bp poly (A)^+ tail. The 423 bp coding region encoded for a 140 aa peptide (preprocalcitonin, MW=15,357 Da). Preprocalcitonin, procalcitonin, calcitonin and katacalcin nucleotide and amino acid sequences comparisons are presented in table 5.1. The deduced aa sequence for equine preproCT was aligned with human, rat, mouse, dog, sheep, salmon, and chicken preproCT sequences, using the BLOSUM 62 scoring matrix in Align plus (Fig. 5.2, table
5.1). There are conserved amino acids in the signal and N-terminal peptides, and mature CT across species. However, there is lack of homology in the carboxyl terminal peptide (CCP, katacalcin). Equine CT signal peptide has 25 amino acids, the N-terminal peptide has 56 amino acids, and katacalcin has 21 aa (MW=2,310 Da). Between the N-terminal peptide and the mature CT there is a dibasic amino acid cleavage site (KR). The predicted equine procalcitonin aa sequence has 115 amino acids (MW=12,507 Da). Equine CT (32aa, MW=3,385 Da) amino acid sequence was compared to human, rat, mouse, dog, salmon, zebrafish, cattle, pig, chicken, sheep, and bullfrog calcitonin, using the BLOSUM 62 scoring matrix in Align plus (Fig. 5.3, table 5.1). A phylogeny dendrogram for calcitonin was constructed using Align plus (Fig. 5.4). The dendrogram has 3 classes, and equine CT was placed in the human, rat, and mouse class.

The signal and N-terminal peptide aa sequences of clone A4 were compared to clones A1 and B1 (Fig. 5.5). There is 100% homology between clones A4 and B1; however the homology between clones A4 (and B1) to clone A1 is 65%, indicating that clone A1 is encoded by a different gene.

Equine CGRP sequence analysis

The cDNA sequences for the signal and N-terminal peptides of clone B1 were identical to clone A4 (Fig. 5.5), which indicated that these clones were derived from the same gene. We named this product equine CGRP-I. Clone B1 (702 bp) contained the complete sequence for equine preproCGRP-I (Fig. 5.6). Equine preproCGRP-I consisted of a 102 bp 5' UTR, a 384 bp coding region, and a 216 bp 3' UTR, including a 25 bp
The 384 bp coding region encoded for a 127 aa peptide (preproCGRP-I, MW=13,863 Da). The deduced aa sequence for mature equine CGRP-I (37aa, MW=3,770 Da) was aligned to CGRP aa sequences from other species (Figs. 5.7-5.8). The greatest homology was found with swine CGRP (59%) and human CGRP-II (59%). The homology with equine clone A1 CGRP was 56%. The signal peptide for equine CGRP-I has 25 aa residues, the N-terminal peptide has 54 aa residues and the C-terminal peptide has 4 aa residues. Between the N-terminal peptide and the mature CGRP-I (37aa) there is a dibasic amino acid cleavage site (KR).

The cDNA sequence for the signal and N-terminal peptides of clone A1 has 65% homology with clones A4 and B1. Clone A1 cDNA likely represents a gene equivalent to the CALC-II gene in humans, which encodes for human CGRP-II. We named the peptide encoded by clone A1 equine CGRP-II. Clone A1 (960 bp) contained the complete sequence for equine preproCGRP-II (Fig. 5.9). Equine preproCGRP-II consisted of a 69 bp 5' UTR, a 390 bp coding region, and a 501 bp 3' UTR, including a 25 bp poly (A)+ tail. The 390 bp coding region encoded for a 129 aa peptide (preproCGRP-II, MW=13,924 Da). The mature equine CGRP-II (37 aa, MW=3,782) aa sequence was aligned to CGRP sequences from other species (Fig. 5.7-5.8). Unlike equine CGRP-I, equine CGRP-II has higher homology to other mammalian and non-mammalian CGRPs (ranging from 81-97% for mammals; Fig. 5.8). The signal peptide for equine CGRP-II has 25 aa residues, the N-terminal peptide has 56 aa residues and the C-terminal peptide has 4 aa residues. A dibasic amino acid cleavage site (KR) is present between the N-terminal peptide and the mature CGRP (37aa), as seen in clones A4 and B1. The C-terminal peptides for clones A1 and B1 are identical, and both are preceded by a 5 aa
cleavage site (GRRRR). A phylogeny dendrogram for CGRPs from different species was constructed using Align plus (Fig. 5.10).

Sequence information was submitted to the NCBI with accession numbers AF249307 (calcitonin), AF257471 (CGRP-I), and AF257470 (CGRP-II).

Northern blot analysis

Northern blot analysis of equine tissues revealed that expression of calcitonin mRNA was restricted to the thyroid gland (Fig. 5.11). In contrast, the expression of equine CGRP-I and CGRP-II mRNA was present in multiple tissues (Fig. 5.11). Expression of CGRP-I was detected in the thyroid gland, hippocampus, pituitary gland, medulla oblongata, dorsal spinal root ganglia, lung, small intestine, and kidney. Expression of CGRP-II was detected in the thyroid gland, basal ganglia, hypothalamus, thalamus, medulla oblongata, spinal cord, dorsal spinal root ganglia, and kidney. The expression of CGRP in the kidney was an unexpected finding.

Discussion

In this study we found three different transcripts encoding for peptides of the equine calcitonin gene family. In humans, calcitonin is encoded by the CALC-I gene (Becker et al., 1996). The CALC-I gene also encodes for CGRP-I (or CGRP-α) by alternative splicing of the primary transcript (Rosenfeld et al., 1981). Other genes of the calcitonin family include CALC-II, encoding for CGRP-II (or CGRP-β), CALC-III (a
pseudogene), and CALC-IV, encoding for amylin (Becker et al., 1996). It has been suggested that CT, CGRP and amylin were encoded by a single gene early in evolution, and that these genes are the result of duplication (Becker et al., 1996). Furthermore, gene duplication, resulting in the CALC-I and CALC-II genes may have resulted in greater biological diversity (Steenbergh et al., 1986).

The equine CT signal and N-terminal peptides were identical to that of equine CGRP-I indicating that clones A4 (CT) and B1(CGRP-I) resulted from alternative splicing of the same primary transcript. Based on the homology of the signal and N-terminal peptides of clone B1 to that of clone A4, we named the deduced aa sequence from clone B1 equine CGRP-I. The degree of homology of mature equine CGRP-I to CGRP in other species is low. The signal and N-terminal peptides of clone A1 have a low homology to that of clones A4 and B1 (65%). We believe that clone A1 is encoded by a different gene (equivalent to human CALC-II). We have named this peptide equine CGRP-II. An unexpected finding was that the mature equine CGRP-II has more homology with CGRPs of other species than equine CGRP-I, and that the homology between equine CGRP-I and CGRP-II was low. The homology between human CGRP-I and human CGRP-II is 91%, while the homology between equine CGRP-I and equine CGRP-II is 56%. We speculate, based on the homology of equine CGRP-II to CGRPs of other species, that CGRP-II in the horse may have assumed many of the functions attributable to CGRP-I in other species (neutransmitter, pain mediator, smooth muscle relaxation), or that equine CGRP has functions other those of a neutransmitter. An example is the expression of equine CGRP-I and CGRP-II in the kidney. To our knowledge, this is the first study that reports expression of a product of the calcitonin
gene family in renal tissue. Bioassay studies would be appropriate to elucidate the
different roles that CGRP may play in the horse. Furthermore, studies on this novel
equine CGRP-I would be of interest from the evolutionary perspective.

This is the first publication on the nucleotide and amino acid sequences of
calcitonin and CGRP in the horse. We have determined that mature equine CT is similar
to CT of other species CT. Equine CGRP-I has low homology to CGRPs of other species,
and we believe, based on the identity of the signal and N-terminal peptides, that equine
CGRP-I is encoded by a gene equivalent to human CALC-I gene.

We are studying the role of the calcitonin gene family in horses with certain
pathological conditions (sepsis, endotoxemia, hypocalcemia). In humans, procalcitonin
may be involved in the pathogenesis of sepsis and inflammation (Oberhoffer et al., 1999).
Measurement of human procalcitonin has been possible by the development of two-site
immunoassays, which require one antibody to calcitonin or to the N-terminal peptide and
a second antibody to the carboxyl terminal peptide (CCP, katacalcin). Unfortunately,
katacalcin is not conserved across species, making the use of human procalcitonin assays
too unreliable to measure equine procalcitonin.

With the cDNA and amino acid sequences for equine preprocalkitonin,
preproCGRP-I and preproCGRP-II available, it is now possible to study the role of the
peptides of the calcitonin gene family in healthy and diseased horses.
Acknowledgments

The authors are grateful to Drs. A. Thiagalingam and B. Nelkin at the Johns Hopkins University Oncology Center for providing us with the sheep calcitonin cDNA (GenBank Accession No. M98053). The authors are also grateful to Drs. D. Groppe, B. LeRoy, V. Richard, R. Sellers, and S. Tannehill-Gregg for their technical assistance.

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colorization and functions of CGRP, related peptides and their receptors. *Neurosci

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Primary structure and bioactivity of bullfrog calcitonin. *Gen Comp Endocrinol* 1997;
107:147–152.
Table 5.1. Nucleotide and amino acid identity (%) of equine preprocalcitonin (preproCT), procalcitonin (proCT), calcitonin (CT) and katacalcin to other species.

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<td>Bullfrog</td>
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NA: Incomplete or no sequence available.
*From aa sequencing.
Figure 5.1. cDNA and deduced amino acid sequences of equine preprocalcitonin (clone A4, GenBank No. AF249307). Numbers on the left indicate nucleotides. Numbers on the right indicate amino acids from the start methionine. The ATG and TAA (*) codons specifying the 423 bp open reading frame and the AATAAA polyadenylation signal are indicated by black boxes. The sequence for the mature calcitonin peptide (32 aa) is underlined. Equine katacalcin amino acid sequence (21 aa) is in a shaded box.
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Figure 5.2. Alignment of the aa sequences of preprocalcitonin from different species. NCBI accession numbers: equine, AF249307; human, M64486; rat, V01229; mouse, X97991; canine, AJ271090; ovine, M98053; salmon, Y00765; and chicken, X03012. Amino acids are shown in one letter code. Amino acid numbers from the start methionine are indicated on the right margin. Dots represent amino acids identical to equine preproCT. Mature calcitonin peptide aa sequence (32aa) is in a shaded box. Katacalcin (CCP) sequence is in a black box.
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Figure 5.3. Alignment of mature calcitonin aa sequences from different species. Amino acids identical to equine CT are presented with dots. Asterisks represent conserved aa (>90%). The overall homology and NCBI accession numbers are shown on the right columns. Amino acids 1, 4-7, 9, 28, and 32 are identical in all known calcitonin sequences. All species have leucine (L) at position 16, except humans, which have phenylalanine (F).
Figure 5.4. Phylogeny dendrogram for calcitonin. The dendrogram was constructed using multi-way alignment with Align plus. Equine calcitonin was placed in the mouse, rat, and human class, with which it has the highest homology. Also shown in Fig. 5.3.
A4  MGFWKFSPLSILVLYQGIIQAAPFRSALESLPDPALPEESRLLAALVDYQVM (60)
B1  ........................................................................................................... (60)
A1  ...G.P.S...AF......C.A.SL...Q.L.S...AS.K.G......A.R (60)

A4  KVRALEQEQTGGAS (75)
B1  ..................... (75)
A1  .TNE......QEMEG (75)

Figure 5.5. Alignment of the signal and N-terminal peptides of clones A4 (CT), B1 (CGRP-I), and A1 (CGRP-II). Dots represent identical amino acids. There is 100% homology between clones A4 and B1, indicating that the gene encoding for these clones is equivalent to the CALC-I gene in humans, which encodes for CT and CGRP-I. Clone A1 sequence is the product of a different gene; the aa homology between clone A1, and clones A4 and B1 is 65%.
Figure 5.6. cDNA and deduced amino acid sequence (127aa) of the equine preproCGRP-I cDNA (clone-B1, GenBank No. AF257471). The single letter aa is shown under the nt sequence. Numbers on the left indicate nucleotides. Numbers on the right indicate amino acids from the start methionine. The ATG and TGA specifying the 384 bp open reading frame and the AATAAA polyadenylation signal are indicated by black boxes. The sequence for the CGRP B1 mature peptide (37 aa) is underlined.
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</table>

**Figure 5.7.** Comparison of the deduced or chemically-determined aa sequences of CGRP (and human amylin) in different species to equine CGRP-I (clone B1). Alignment was performed with Align plus software. Nucleotide and amino acid sequences were obtained from the NCBI (http://www.ncbi.nlm.nih.gov). Asterisks represent conserved aa (>90%) among species. Dots represent amino acids identical to equine CGRP-I (clone B1). Amino acids DF (14-15) are only present in chicken, bullfrog, phyllomedusa, and salmon. The degree of homology (%) of clone B1 to other species is low (<59%). Amino acids 2-5, 7, 9, 11-13, 16, 27, 30, and 33 are identical between equine CGRP-I (clone B1) and human amylin (35%).
### Table 5.8. Alignment of the deduced or chemically determined aa sequences of CGRP (and human amylin) in different species compared to equine CGRP-II (clone Al).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>Homology</th>
<th>NCBI No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>SCNTATCVTHRLAGLLSRSVGVVNSFPTDVGSEAF</td>
<td>97</td>
<td>AP257470</td>
</tr>
<tr>
<td>Swine</td>
<td></td>
<td>97</td>
<td>227702</td>
</tr>
<tr>
<td>Ovine</td>
<td></td>
<td>94</td>
<td>P30880</td>
</tr>
<tr>
<td>Rat I</td>
<td></td>
<td>94</td>
<td>P30881</td>
</tr>
<tr>
<td>Rat II</td>
<td></td>
<td>89</td>
<td>L29188</td>
</tr>
<tr>
<td>Human-1</td>
<td>A.D................</td>
<td>86</td>
<td>X15943</td>
</tr>
<tr>
<td>Human-2</td>
<td>A...................</td>
<td>81</td>
<td>P10286</td>
</tr>
<tr>
<td>Bullfrog</td>
<td>A...................</td>
<td>78</td>
<td>P31888</td>
</tr>
<tr>
<td>Chicken</td>
<td>A...................</td>
<td>72</td>
<td>S40497</td>
</tr>
<tr>
<td>Salmon I</td>
<td>A...................</td>
<td>75</td>
<td>U71287</td>
</tr>
<tr>
<td>Salmon IV</td>
<td>A...................</td>
<td>59</td>
<td>P81564</td>
</tr>
<tr>
<td>Human Amylin B1</td>
<td>S.L..............SA.SMAM..LL.EM.FKVS</td>
<td>56</td>
<td>AP257471</td>
</tr>
<tr>
<td>Cloned Bl</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unlike equine CGRP-I, the aa sequence of equine CGRP-II is more conserved when compared to other species. Asterisks represent conserved aa (>90%). Identical amino acids to that of equine CGRP-II (Al) are presented in dots. Amino acids 2-7, 9, 11-13, 16, 19, 30, and 32-34 are identical between equine CGRP-II (clone Al) and human amylin (43%). The homology at the N-terminus may be responsible for some overlap in function between amylin and CGRP.
Figure 5.9. cDNA and deduced amino acid sequence (129aa) of the equine CGRP-II cDNA (clone A1, GenBank No. AF257470). The single letter aa is shown under the nt sequence. Numbers on the left indicate nucleotides. Numbers on the right indicate amino acids from the start methionine. The ATG and TGA specifying the 390 bp open reading frame and the polyadenylation signal are indicated by black boxes. The sequence for the CGRP mature peptide (37 aa) is underlined.
Figure 5.10. Phylogeny dendrogram for CGRP. The dendrogram was constructed with the Align plus software. Two major branches representing mammalian and low vertebrate CGRPs are present. One additional branch representing clone B1 (equine CGRP-I) suggest an early divergence of this gene when compared to clone A1 (equine CGRP-II).
Figure 5.11. Tissue-specific expression of equine calcitonin, CGRP-I, and CGRP-II mRNA. The blots were probed as described in section 2.4. (A) Ethidium bromide stained agarose gel to demonstrate RNA loading and integrity. The tissues are 1=thyroid gland, 2=basal ganglia, 3=hippocampus, 4=rostral brain, 5=caudal cortex, 6=hypothalamus, 7=thalamus, 8=pituitary gland, 9=medulla oblongata, 10=cerebellar vermis, 11=cerebellar hemisphere, 12=spinal cord, 13=dorsal root ganglia, 14=lung, 15=small intestine, 16=adrenal gland, 17=parathyroid gland, 18=spleen, 19=liver, 20=kidney. Phosphorimages for equine calcitonin (B), CGRP-I (C), and CGRP-II (D).


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Song SK, Karl IE, Ackerman JJ, Hotchkiss RS. Increased intracellular Ca^{2+}: a critical link in the pathophysiology of sepsis?. *Proc Natl Acad Sci USA* 1993; 90:3933–3937.


