Endocrine, *vol. 9, no. 3, 293–301, December 1998* 0969–711X/98/8:293–301/$10.25

Stimulation of Calcitonin Secretion by Calcium Receptor Activators

Evaluation Using a New, Highly Sensitive, Homologous Immunoradiometric Assay for Rat Calcitonin

Jeffrey R. Lavigne,[1] Richard J. Zahradnik,[1] Rebecca L. Conklin,[2] Lyssa D. Lambert,[2] Mary A. Logan,[2] Ashutosh Parihar,[2] and John Fox[2]

[1]*Immutopics, Inc., San Clemente, CA; and* [2]*NPS Pharmaceuticals, Inc., Salt Lake City, UT*

Current rat calcitonin immunoassays use human calcitonin antisera, and suffer from poor sensitivity, long incubation periods, nonspecific interferences, and unreliability. The homologous immunoradiometric assay (IRMA) for rat calcitonin described here overcomes these problems. Overnight incubation yields a detection limit of 0.4 pg/mL, a standard curve that is linear to >1800 pg/mL, and intra- and interassay coefficients of variation of <7%. Gel filtration chromatography of rat plasma and rat medullary thyroid carcinoma 44-2 cell media showed that the vast majority of immunoreactivity coeluted with calcitonin standard. In 44-2 cells, increasing extracellular Ca^{2+} concentration or incubation with the calcimimetic compound NPS R-467 markedly increased calcitonin secretion. Plasma calcitonin levels were elevated in rats anesthetized with ketamine/xylazine and in conscious rats with chronic renal insufficiency. Calcitonin levels decreased following EGTA-induced hypocalcemia and were undetectable after thyroparathyroidectomy. In normal conscious rats, plasma calcitonin levels averaged 3–5 pg/mL and increased up to 100-fold following calcium (Ca) infusion or NPS R-467 administration. The assay also quantified calcitonin in plasma of normal and Ca-injected mice. This assay has revealed that plasma calcitonin levels in normal rats are much lower than the detection limits of most existing assays, but can increase by 100-fold on activation of the C-cell Ca^{2+} receptor.

Key Words: Rat; calcitonin; immunoradiometric assay; calcimimetics; rat medullary thyroid carcinoma 44-2 cells.

*The rat calcitonin assay described in this paper is available as a kit from Immutopics, Inc., 929 Calle Negocio, Suite A, San Clemente, CA 92673.

Received September 18, 1998; Revised October 9, 1998; Accepted October 9, 1998.

Author to whom all correspondence and reprint requests should be addressed: Dr. John Fox, NPS Pharmaceuticals, Inc., 420 Chipeta Way, Salt Lake City, UT 84108. E-mail: jfox@npsp.com

Introduction

Plasma calcium (Ca) homeostasis is maintained largely by the interplay of three hormones, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$), and calcitonin. PTH is secreted in response to a fall in plasma ionized calcium (Ca^{2+}) levels, and acts to restore normocalcemia by increasing the renal tubular reabsorption of Ca^{2+}, bone resorption, and the renal synthesis of $1,25(OH)_2D_3$. Hypercalcemia, on the other hand, results in an increased secretion of calcitonin from the parafollicular cells (C-cells) of the thyroid. Calcitonin acts to restore normocalcemia and inhibits osteoclast-mediated bone resorption *(1–3)*. Although calcitonin clearly plays an important role in systemic Ca^{2+} homeostasis in many terrestrial vertebrates *(4,5)*, the physiological importance of this action in humans is uncertain *(1–3,6)*. Nevertheless, calcitonin has found a place in the treatment of diseases, such as Paget's disease and osteoporosis, that are characterized by high bone turnover *(2,3)*.

The effects of extracellular Ca^{2+} on the secretion of PTH and calcitonin are mediated by a cell-surface Ca^{2+} receptor *(7–9)*. These Ca^{2+} receptors might therefore serve as targets for new drugs that can directly alter the secretion of PTH and/or calcitonin *(10)*. Scientists at NPS Pharmaceuticals have discovered compounds that act as positive allosteric modulators at the Ca^{2+} receptor *(10,11)*. These compounds, termed type II calcimimetics and typified by NPS R-467 and NPS R-568, are potent and selective inhibitors of PTH secretion in vitro *(11)*, and when administered orally, rapidly lower plasma levels of PTH and Ca^{2+} in rats *(12)*. NPS R-568 also elevates plasma calcitonin in rats, but does so only at doses about 40 times higher than those that suppress PTH levels *(13)*.

The mechanism responsible for the differing potency of NPS R-568 at parathyroid and C-cell Ca^{2+} receptors is unknown. It does not appear to result from differences in receptor structure, because the mRNA sequences are identical in both tissues, and Western blot analysis indicates that the expressed proteins are also very similar *(9)*. We

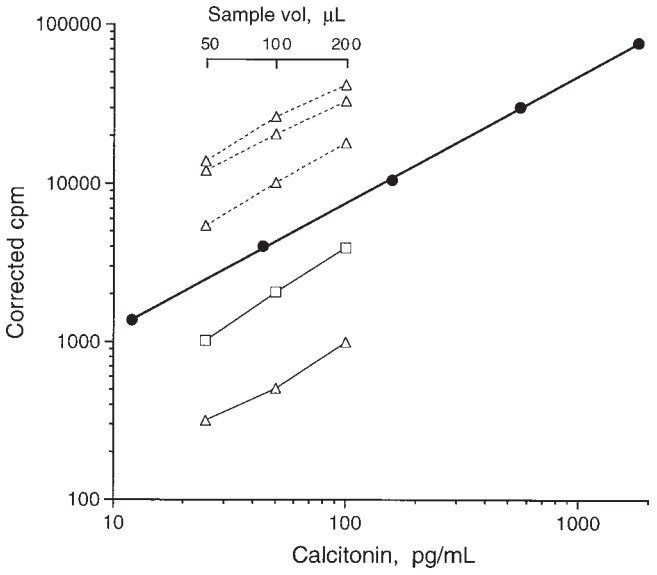

Fig. 1. Typical standard curve from rat calcitonin IRMA. Corrected cpm values were obtained for nonzero standards and samples by subtracting 484 cpm, the average cpm of the assay zero-standard. The standard curve was linear to 1800 pg/mL. Immunoreactivity in samples of plasma from rats with high (△---△) and normal (△—△) calcitonin levels, and culture media from rat MTC 44-2 cells (□—□), diluted to 200 µL with assay zero-standard, paralleled the standard curve.

thought it possible that the apparently lower in vivo potency of NPS R-568 at C-cells could be an artifact of the radioimmunoassay used to quantify plasma levels of calcitonin in rats. Like the vast majority of assays used to measure rat calcitonin *(14,15)*, our immunoassay used an antiserum to human calcitonin *(16)*, and suffered from inadequate sensitivity and nonspecific interferences. Therefore, the markedly lower potency of NPS R-568 at the C-cell may result from a failure of the assay to detect small increases in calcitonin levels occurring with lower doses of the compound.

To overcome problems associated with assay insensitivity and unreliability, and to determine whether the differences in the potency of calcimimetics at parathyroid and C-cells are an artifact, we have developed a highly sensitive, homologous, two-site immunoradiometric assay (IRMA) for rat calcitonin. Extensive validation and characterization of this new assay have revealed that calcitonin levels in normal rats are markedly lower than indicated by earlier assays, decrease appropriately in response to hypocalcemia and thyroidectomy, and increase substantially following activation of the Ca^{2+} receptor with extracellular Ca^{2+} or NPS R-467.

Results

Assay Performance and Characteristics

When plotted on a log-log scale, the standard curve was linear up to the highest standard tested (1800 pg/mL) (Fig. 1). In the most recent six assays, the correlation coefficient averaged 0.9994 (range, 0.9989–0.9999), the slope averaged 0.822 (range, 0.797–0.844), and the standard error of the slope averaged 0.019 (range, 0.009–0.028). The "hook effect," when the measured counts per minute (cpm) decreased below that of the highest standard, was not observed until calcitonin concentrations exceeded 300,000 pg/mL (not shown). Samples of rat plasma with normal and elevated calcitonin levels, and culture media from rat medullary thyroid carcinoma (MTC) 44-2 cells paralleled the standard curve when diluted with assay zero standard (Fig. 1). Two standard deviations of the mean cpm of the zero standard were used to calculate the assay detection limit. In those recent six assays, the detection limit averaged 0.44 pg/mL (range 0.28–0.60 pg/mL), and the intra- and interassay coefficients of variation for an internal reference standard (27.1 pg/mL) averaged 4.3 and 7.2%, respectively. Cross-reactivity of human calcitonin in the assay was 12.5%; for porcine, salmon, and chicken calcitonin, it was <0.001%. Processing of samples of rat blood to produce either serum or heparinized plasma yielded very similar calcitonin values (data not shown).

Chromatography

Circulating immunoreactive calcitonin is often heterogeneous in humans *(3,17,18)*. To elucidate the nature of the immunoreactivity that is detected by the new assay, two samples of rat plasma and one sample of media from rat MTC 44-2 cells were subjected to size-exclusion chromatography. Although small peaks of immunoreactivity eluted earlier than the standard, a single major peak of immunoreactivity that coeluted with synthetic rat calcitonin-(1-32) standard was observed in all three samples. For the two plasma samples, the major peak accounted for 94–97% of the total immunoreactivity eluted from the column. For the rat MTC 44-2 cell media, the major peak accounted for 87% of the total immunoreactivity; in this sample, three small, but distinct additional peaks of immunoreactivity were observed (Fig. 2).

In Vitro Studies in Rat MTC 44-2 Cells

Rat MTC 44-2 cells express abundant levels of the Ca^{2+} receptor *(9)* and, therefore, are useful in the identification of calcimimetic compounds that stimulate calcitonin secretion. Preliminary studies showed that the maximum calcitonin secretory response induced by increasing extracellular Ca^{2+} levels usually occurred when the MTC 44-2 cells were 70–80% confluent. In these cells, increasing extracellular Ca^{2+} levels usually increased the secretion of calcitonin by three- to fourfold. In the first experiment, the EC_{50} for stimulation of calcitonin secretion by Ca^{2+} was 1.2 ± 0.1 mmol/L (Fig. 3A). In a second experiment, the addition of NPS R-467 to incubation media containing 1.2 mmol/L Ca^{2+} caused a concentration-dependent increase in calcitonin secretion. The EC_{50} for stimulation of calcitonin secretion by NPS R-467 was 1.4 ± 0.2 µmol/L (Fig. 3B).

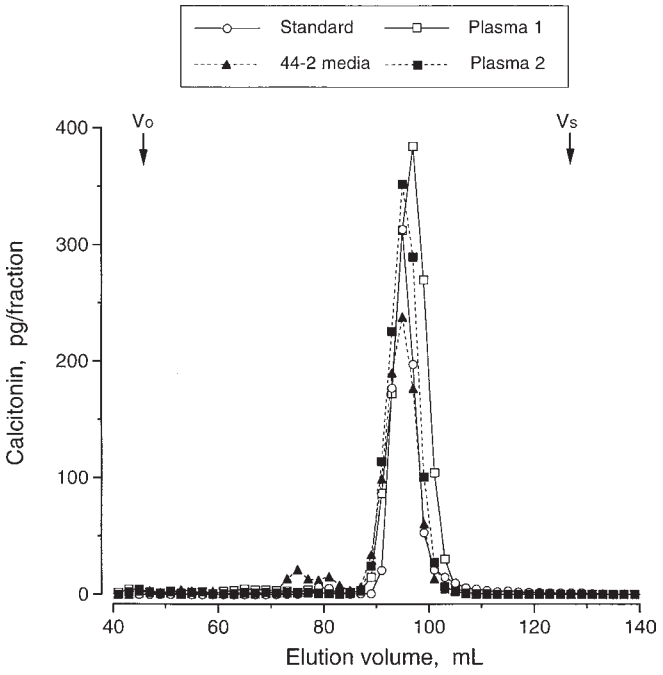

Fig. 2. Gel-filtration chromatography (1.5 x 80 cm column of Sephadex G-50, superfine) of 2-mL samples of rat plasma collected at 1 min after an iv injection of Ca gluconate (calcitonin levels in plasmas 1 and 2 = 885 and 721 pg/mL, respectively) and 2 mL culture media from rat MTC 44-2 cells (calcitonin level = 642 pg/mL). Although other small peaks eluted earlier, the vast majority of the immunoreactivity coeluted with synthetic rat calcitonin-(1-32) standard. Vo and Vs are void volume and salt volume of column, respectively.

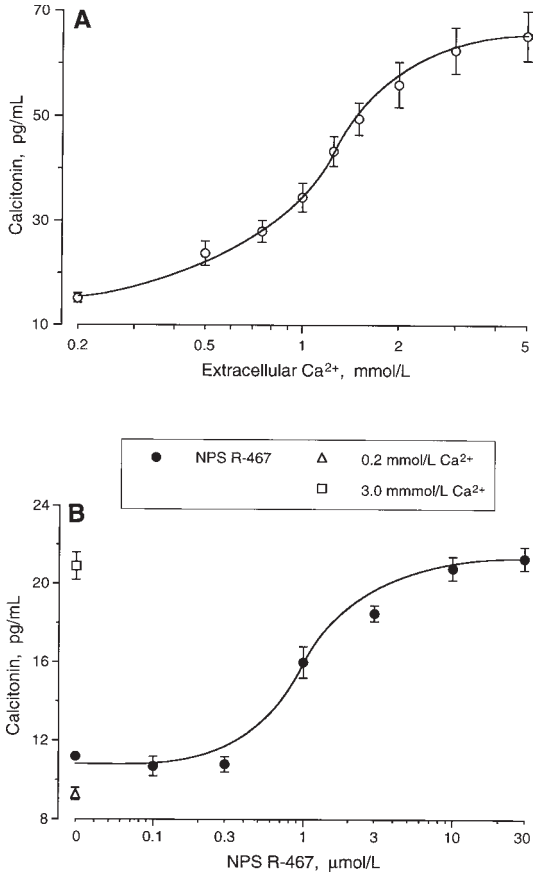

Fig. 3. Stimulation of calcitonin secretion by extracellular Ca^{2+} **(A)** or the calcimimetic compound NPS R-467, tested at 1.2 mmol/L Ca^{2+} **(B)**, in rat MTC 44-2 cells. Values are means ± SE of triplicate assays at each concentration.

In Vivo Studies in Rats: Effects of Anesthesia and Chronic Renal Insufficiency

It has been reported that the plasma levels of calcitonin are elevated in anesthetized rats *(15)*. To assess this observation using the present assay, we measured the plasma levels of calcitonin in conscious and anesthetized rats. Calcitonin levels were elevated fivefold in rats anesthetized with ketamine and xylazine. The hypercalcitoninemia was not caused by hypercalcemia, since plasma Ca^{2+} levels were unchanged (Table 1). Moreover, neither ketamine nor xylazine by itself or together exhibited detectable activity when tested in HEK 293 cells stably transfected with the Ca^{2+} receptor (unpublished observations).

Calcitonin levels are often elevated in patients with chronic renal insufficiency (CRI) *(19)*. We investigated whether uremic rats exhibited similar increases in plasma calcitonin. Conscious 5/6 Nx rats had modest elevations in blood urea nitrogen (BUN) levels but, because they were fed a high phosphorus diet, were significantly hypocalcemic and hyperphosphatemic with moderate-to-severe secondary hyperparathyroidism. Despite the hypocalcemia, the plasma levels of calcitonin were elevated 2.7-fold in the uremic animals (Table 1).

Table 1
Plasma Calcitonin Levels are Elevated in Anesthetized Normal Rats and in Conscious 5/6 Nephrectomized Rats with Chronic Renal Insufficiency[a]

Parameter	Conscious	Anesthetized
Calcitonin (pg/mL)	5.9 ± 0.6	29.6 ± 3.6[b]
Ca^{2+} (mmol/L)	1.38 ± 0.01	1.39 ± 0.01
n	26	10
	Sham-operated	**5/6 Nephrectomized**
BUN (mg/dL)	19 ± 1	46 ± 2[b]
Ca^{2+} (mmol/L)	1.38 ± 0.01	1.29 ± 0.01[b]
Phosphate (mmol/L)	1.86 ± 0.03	3.11 ± 0.14[b]
PTH (pg/mL)	33 ± 2	305 ± 28[b]
Calcitonin (pg/mL)	5.6 ± 0.6	15.1 ± 1.6[b]
n	50	46

[a]Conscious and anesthetized normal rats were fed standard chow; sham-operated and 5/6 nephrectomized rats were fed a 0.6% Ca, 1.2% P diet for 6 wk after surgery. Rats were anesthetized with ketamine/xylazine injected im. Samples were collected from the abdominal aorta via a chronic catheter in conscious rats or by direct puncture of the aorta at about 5 min after the injection in anesthetized rats. [b]$p < 0.05$.

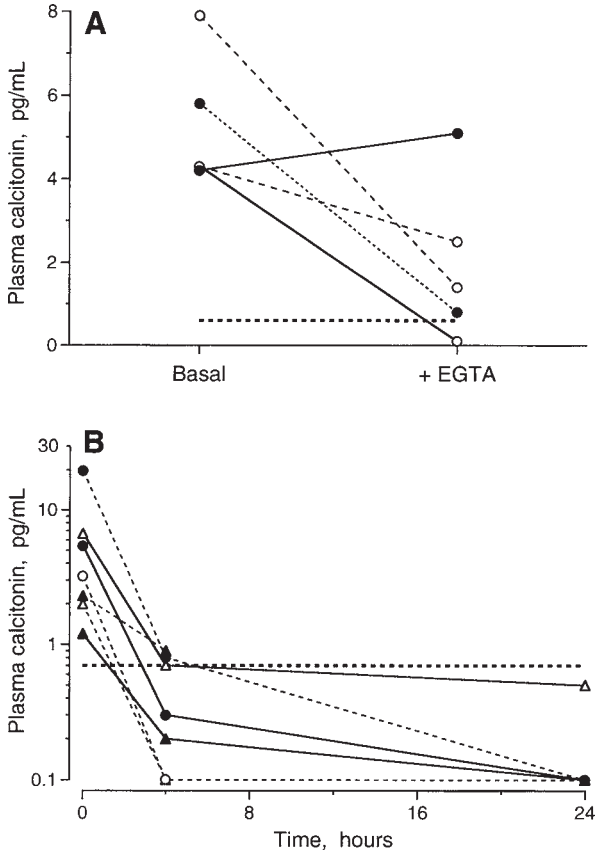

Fig. 4. Changes in plasma levels of calcitonin at 15 min after the iv injection of EGTA **(A)** and after a total thyroparathyroidectomy **(B)** of normal rats. In each panel, each line represents a separate animal, and the bold horizonal dashed line indicates the assay limit of detection.

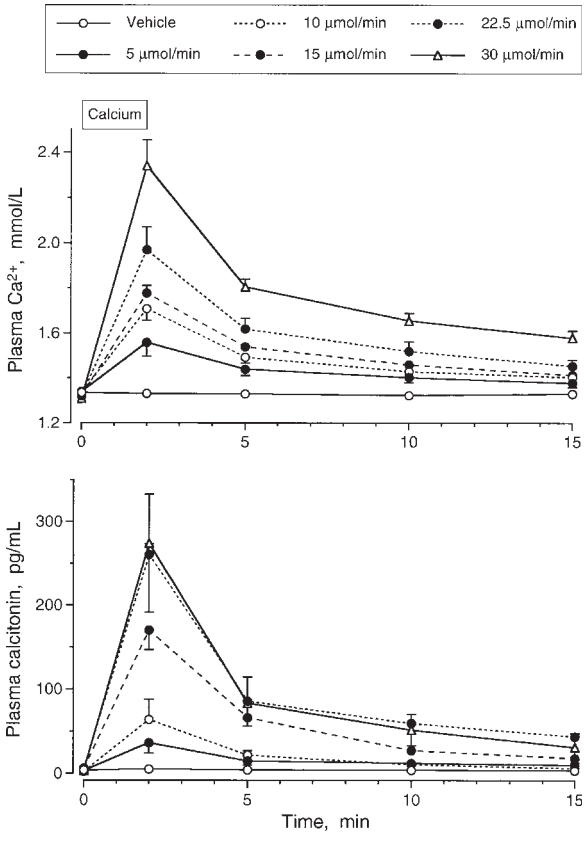

Fig. 5. Effects of iv infusion of Ca gluconate (5–30 µmol/min for 2 min) on plasma levels of Ca^{2+} and calcitonin in normal conscious rats. Values are means ± SE; n = 5–10/group.

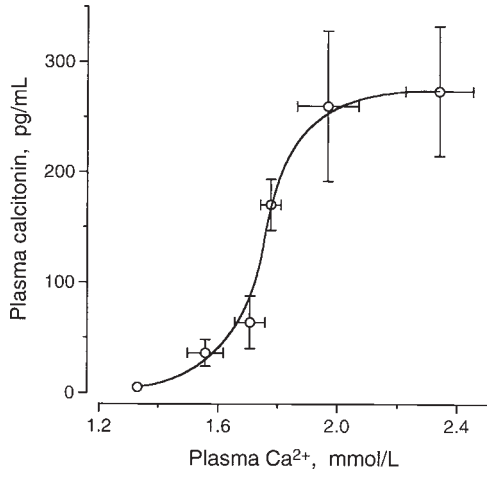

Fig. 6. Plasma Ca^{2+} vs calcitonin concentration–response curve in normal rats. Values are means ± SE and are taken from the 2-min time-point of Fig. 5. The calculated EC_{50} is 1.71 mmol/L Ca^{2+}.

Effects of Hypocalcemia or Thyroparathyroidectomy

One of the limitations of current immunoassays is their inability to detect consistently decreases in calcitonin levels from normal. We therefore assessed whether the new assay could quantify decreases in calcitonin levels induced by hypocalcemia and by thyroparathyroidectomy. The intravenous (iv) infusion of ethylene glycol-bis-β-aminoethyl-ether-N,N,N',N'-tetraacetic acid (EGTA) in five, normal conscious rats decreased plasma Ca^{2+} levels from 1.42 ± 0.01 to 0.92 ± 0.11 mmol/L at 15 min after the start of the infusion. Although the average change in plasma calcitonin was not statistically significant, calcitonin levels decreased substantially in four of the five animals. However, calcitonin levels were reduced below the detection limit of the new assay in only one rat (Fig. 4). In contrast, and despite the anesthesia, calcitonin levels rapidly became undetectable in all rats subjected to a thyroparathyroidectomy (Fig. 4).

Effects of Hypercalcemia

The 2-min iv infusion of Ca gluconate caused a dose-dependent increase in the plasma levels of Ca^{2+} and a corresponding significant increase in plasma calcitonin concentration at all infusion rates (Fig. 5). In the vast majority of the animals, plasma levels of both Ca^{2+} and calcitonin were maximal at the end of the infusion and declined rapidly from the peak concentrations when the infusion was discontinued. Despite the greater increase in plasma Ca^{2+} levels in rats that received the highest rate of Ca infusion (30 µmol/min), the average plasma calcitonin

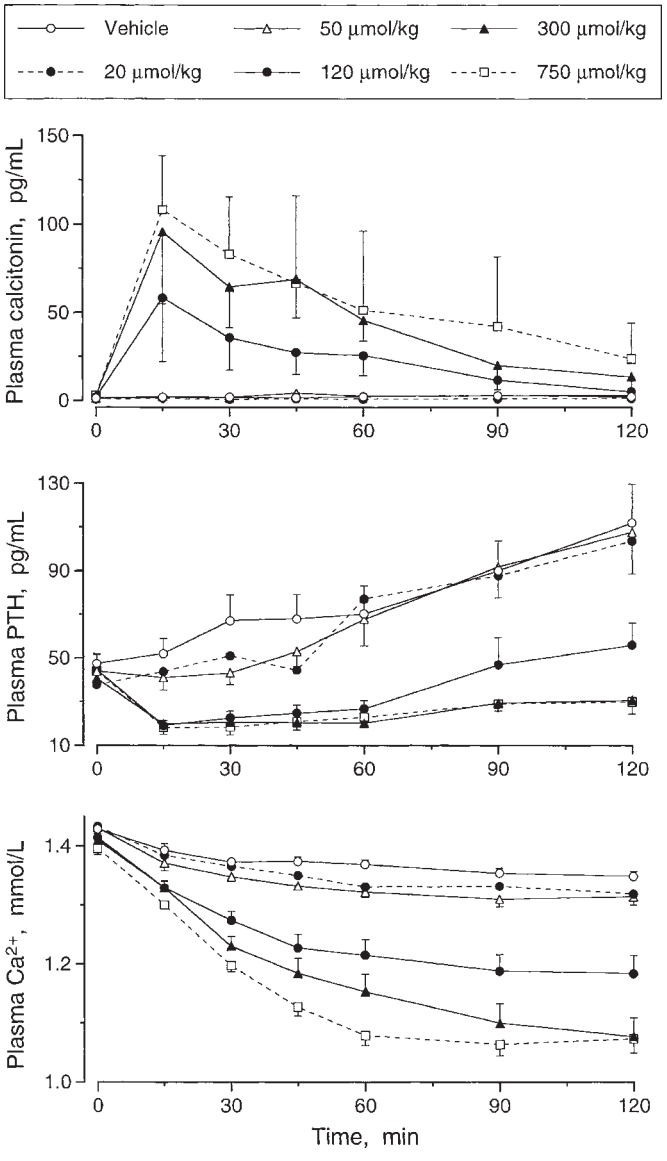

Fig. 7. Effects of oral administration of NPS R-467 on plasma levels of calcitonin, PTH, and Ca^{2+} in normal rats. Values are means ± SE; n = 7–8/dose.

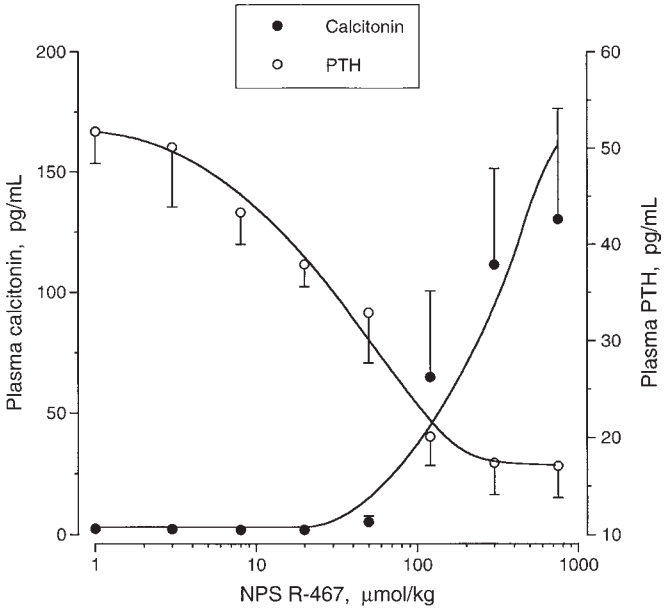

Fig. 8. Dose–response to orally administered NPS R-467 in normal rats. The calcitonin data represent the highest values, and PTH data the lowest values at 15–45 min after dosing in each rat (*see* Fig. 7). Values are mean ± SE, n = 7–8/dose.

response (90-fold) was essentially the same in rats receiving either the 22.5 or 30 μmol/min Ca infusion. The EC_{50} for elevation of calcitonin levels by plasma Ca^{2+} was 1.71 ± 0.09 mmol/L (Fig. 6).

Effects of the Calcimimetic NPS R-467

Plasma levels of calcitonin increased following the oral administration of NPS R-467. Calcitonin levels were maximal at 15–45 min after dosing in all rats studied, although the magnitude of the increase varied considerably between rats. Because of this variability, doses of <300 μmol/kg did not significantly affect calcitonin levels, although the 120 μmol/kg dose increased calcitonin levels on average 25-fold to 65 ± 36 pg/mL (Figs. 7 and 8). Calcitonin levels declined rapidly from the peak and were not significantly elevated during the last hour of the experiment. Because the plasma calcitonin response did not plateau at the highest dose of NPS R-467 tested (750 μmol/kg), it was not possible to quantify the ED_{50} accurately (Fig. 8). Fixing the maximum plasma calcitonin level in the Levenberg-Marquardt equation at 265 pg/mL, the maximum level observed following Ca infusion (Fig. 5), yielded an ED_{50} for elevation of calcitonin levels by NPS R-467 of 580 ± 190 μmol/kg.

Doses of NPS R-467 ≥20 μmol/kg caused significant decreases in the plasma levels of PTH at 15–45 min after dosing (Figs. 7 and 8). PTH levels remained maximally suppressed throughout the 2-h experiment with the 300 and 750 μmol/kg doses, but returned to near control levels more rapidly at the lower doses. The ED_{50} for suppression of PTH levels by NPS R-467 was 36 ± 17 μmol/kg (Fig. 8).

The oral administration of NPS R-467 also resulted in a rapid decrease in plasma Ca^{2+} levels (Fig. 7). Doses of NPS R-467 ≥20 μmol/kg caused significant decreases in plasma levels of Ca^{2+} below those in animals that received vehicle. A nadir in Ca^{2+} levels occurred in most rats between 1 and 2 h after dosing. Plotting the minimum plasma Ca^{2+} level in each rat vs the dose of NPS R-467 yielded an ED_{50} of 110 ± 13 μmol/kg.

Effects of Ca Injection in Mice

The amino acid sequence of mouse calcitonin differs from that of rat at only one residue *(20)*. To assess whether the new assay would detect basal or stimulated calcitonin levels in the mouse, saline or Ca (500 μmol/kg) was injected intraperitoneally (ip) in normal adult mice. At 10 min after the injection, calcitonin levels were 5.6 ± 0.4 pg Eq rat calcitonin/mL (n = 3) in the mice that received saline and

128 ± 45 pgEq/mL (range, 39–252 pg/mL; $n = 4$) in the mice injected with Ca ($p < 0.05$).

Discussion

This article describes the development, validation, and extensive practical use of a new, highly sensitive, and reliable homologous IRMA for rat calcitonin. The detection limit of this assay (0.4 pg/mL) is 40–60 times lower than in the one we used previously *(16)*. Unlike previous immunoassays, this new IRMA has sufficient sensitivity to detect decreases easily in plasma levels of calcitonin in normal rats. Moreover, the quantification of decreases in calcitonin levels is possible despite the discovery that normal plasma levels of calcitonin in rats (about 3–5 pg/mL) are lower than the detection limit of most other immunoassays.

Although the antisera used in this IRMA were raised against the rat hormone, calcitonin immunoreactivity was also easily detected in mouse plasma, both before and after stimulation of calcitonin secretion by Ca injection. Using this assay, we have also demonstrated that calcitonin levels are elevated in the fetuses of wild-type, heterozygous, and homozygous Ca^{2+} receptor knockout mice when compared with normal mice *(21)*. However, because mouse standards are not available, we were unable to determine the cross-reactivity of mouse calcitonin in this assay.

Although a rat calcitonin radioimmunoassay was developed over 20 years ago, it could not detect the hormone in the peripheral blood of rats *(22)*. Thus, circulating levels of calcitonin in rats have usually been quantified by immunoassays that use antisera to human calcitonin *(14–16)*. These heterologous assay systems have been possible, because in contrast to the substantial sequence differences in calcitonin between most species, rat and human calcitonin differ at only 2 of the 32 amino acids *(2,23)*. Despite this sequence similarity, rat calcitonin immunoassays based on human calcitonin antisera still suffer from poor sensitivity, despite incubations of many days at 4°C, and poor reliability because of nonspecific interferences. All of these problems have been overcome with this new assay. Assay sensitivity has been enhanced dramatically and is achieved with a simple overnight incubation at room temperature. Moreover, the assay standard curve has a very wide and linear dynamic range (0.4–1800 pg/mL), with the upper limit exceeding by almost twofold the highest calcitonin level we have observed in a rat (950 pg/mL). The only potential concern for studies that involve repetitive blood sampling in rats or experiments in mice is the plasma sample volume requirement (200 μL). However, we have shown that samples can be diluted up to fourfold with assay zero-standard and still generate accurate results.

We noted almost 15 years ago that as assay methodology continued to improve, what was considered a normal calcitonin level in humans also continued to decrease *(24)*. In a tongue-in-cheek comment, we speculated that if the trend continued, calcitonin would disappear in 1984. Although this did not of course happen, the recent introduction by Nichols Institute Diagnostics of a highly sensitive (detection limit, 0.7 pg/mL) immunochemiluminometric assay for human calcitonin has continued this trend *(19)*. Normal calcitonin levels in humans are considerably lower than the detection limit of older immunoassays and are very similar to those revealed by the studies in rats reported here.

Gel-filtration chromatography of rat plasma and rat MTC 44-2 cell media showed that the vast majority of the immunoreactivity coeluted with a rat calcitonin standard. The heterogeneity of circulating calcitonin in humans has been extensively investigated in patients with MTC. The earlier-eluting higher molecular weight immunoreactivity is mainly due to procalcitonin and products of its cellular processing, although calcitonin dimers may also be present *(3)*. There is also evidence that the calcitonin monomer can associate with plasma proteins by a thiol–disulfide interchange *(25)*. Abnormal secretion of immature forms of calcitonin may be a characteristic of MTC, since several small peaks of immunoreactivity eluted before the calcitonin standard when media from rat MTC 44-2 cells were chromatographed. However, we cannot be certain if the failure to detect greater levels of the larger forms of calcitonin in normal rat plasma is because none are present or because their level of crossreactivity in this assay is low.

Other studies designed to evaluate the new assay showed that ketamine/xylazine anesthesia markedly increased calcitonin levels in normal rats without affecting plasma Ca^{2+} levels. Anesthesia-induced hypercalcitoninemia in rats had been observed previously when older assays were used *(15)*. The mechanism responsible for this phenomenon is unclear, but it appears not to be related to direct agonist actions of the anesthetic agents at the Ca^{2+} receptor. A similar Ca^{2+}-independent increase in PTH levels has also been seen in anesthetized rats *(26)*. Because the administration of ketamine and xylazine often results in a profound decrease in blood pressure *(27)*, the increase in PTH and calcitonin levels may be caused by decreased clearance of the hormones from the circulation rather than by stimulation of secretion. Plasma levels of calcitonin were also elevated in rats with chronic renal insufficiency despite the presence of significant hypocalcemia. It is possible that the elevated plasma calcitonin is contributing to the hypocalcemia. Elevated calcitonin levels have also been observed in dialysis patients *(19)*. The kidney is the major site of clearance of calcitonin from the circulation *(28)* and decreased renal function might, therefore, be expected to increase calcitonin levels. However, the persistent hypercalcitoninemia in uremic rats remains puzzling, because the hypocalcemia should provide feedback to inhibit calcitonin secretion.

Further studies in normal rats showed that plasma calcitonin levels increased substantially when calcitonin secretion was stimulated by iv Ca gluconate infusion. The

average maximum calcitonin level achieved (~300 pg/mL) was very similar whether quantified using the old radioimmunoassay *(13,16)* or the new IRMA, but because of the lower baseline levels of calcitonin, the magnitude of the increase in calcitonin levels was greater (100- vs 10-fold) with the new assay.

Previous studies showed that there was about a 40-fold difference in the potency of the calcimimetic compound NPS R-568 in reducing PTH levels than increasing calcitonin in normal rats *(12,13)*. This unexpected finding occurred despite the fact that the Ca^{2+} receptor protein appears to be identical on parathyroid cells and C-cells *(9)*. We postulated that this could have been owing, at least in part, to the relatively insensitive radioimmunoassay used previously to quantify changes in plasma calcitonin. Although it was not possible to determine accurately the ED_{50} for elevation of calcitonin levels following oral administration of the closely related calcimimetic NPS R-467, the results strongly suggested that this difference in potency was maintained when calcitonin levels were measured with the new assay. Other possible explanations for the dissimilar potencies include differing receptor densities in the two cell types, since loss of the Ca^{2+} receptor in cultured bovine parathyroid cells is associated with a markedly impaired sensitivity to Ca^{2+} *(29)*; however, immunocytochemistry has not revealed any gross quantitative differences in expression levels in parathyroid and C-cells (unpublished data). Alternatively, the different postreceptor mechanisms linking the Ca^{2+} receptor to hormone secretion *(30,31)* may be coupled with different efficiencies in the two cell types.

The relatively recent development of a highly sensitive IRMA that is specific for the biologically active region of rat PTH has dramatically improved the reliability of PTH measurements in the rat *(12,13,26,32,33)*. In particular, the ability to quantify decreases in PTH levels from normal was not consistently possible prior to the development of that assay. We expect that this new calcitonin IRMA will similarly revolutionize the measurement of calcitonin in the rat. Use of this assay to quantify calcitonin secretion both in vitro using rat MTC 44-2 cells and in vivo will facilitate the identification of small organic compounds that in contrast to calcimimetics like NPS R-467 and NPS R-568, may act as selective activators of the C-cell Ca^{2+} receptor. By selectively stimulating the secretion of calcitonin, such compounds could be useful therapeutic agents in the treatment of metabolic bone diseases associated with high bone turnover.

Materials and Methods

Assay Development and Procedure

Polyclonal antibodies to rat calcitonin were prepared by immunization of goats with synthetic rat calcitonin-(1-32) (Bachem, Torrance, CA) conjugated to keyhole limpet hemocyanin with glutaraldehyde *(34)*. Monoclonal antibodies (MAbs) recognizing the rat calcitonin molecule were obtained from commercial sources (Biogenesis, Sandown, NH). The polyclonal antibodies were immunopurified via conventional affinity chromatography procedures to various peptide binding sites on the rat hormone. Each antibody was immobilized by adsorption onto 6-mm diameter polystyrene beads (Craig Technologies, Folcroft, PA) and also radioiodinated by use of a modified chloramine-T procedure *(35)* to a specific activity of approx 20 Ci/g. Pairs of MAbs and purified polyclonal antibodies were then systematically assessed in an immunometric-type assay for optimum binding characteristics. The antibody pair showing the greatest affinity and specificity for the intact rat hormone in this sandwich-type screening assay was selected for use in the final assay. The final system paired a MAb with an affinity-purified polyclonal antibody for capture and detection, respectively.

Synthetic rat calcitonin-(1-32) standards, internal reference standards, and sample (200 μL) were pipeted into 12 × 75 mm tubes followed by ^{125}I-labeled rat calcitonin antibody (100 μL). The tubes were rapidly mixed, and one antibody-coated bead was added to each tube. Following an overnight incubation at room temperature, the contents of the tubes were aspirated, and the beads washed three times by dispensing 2 mL of a wash solution into each tube and completely aspirating the contents. The washed beads were counted in a gamma counter for 5 min. We routinely distributed at least six zero-standard tubes throughout each ~100 tube assay. The average cpm of these zero-standards were subtracted from the cpm of each calcitonin standard and sample. The corrected cpm were plotted against rat calcitonin standard concentration to yield the standard curve. Sample calcitonin concentrations were calculated using a Microsoft Excel spreadsheet.

Chromatography

Samples (2 mL) of rat plasma collected at 1 min after an iv injection of Ca gluconate, media from rat MTC 44-2 cells, and a rat calcitonin assay standard were applied to an 80 × 1.5 cm column of Sephadex G-50 (superfine) equilibrated and eluted with acetic acid (10 mmol/L) at a rate of 0.25 mL/min. ^{125}I-labeled rat PTH antibody and free ^{125}I were added to each sample to mark the void volume and salt volume of the column, respectively. Fractions (2 mL) were collected, counted for ^{125}I radioactivity, and evaporated to dryness in a vacuum centrifuge. The dried eluants were reconstituted with 200 μL of the assay zero-standard and assayed directly for calcitonin content.

In Vitro Studies in Rat MTC 44-2 cells

Rat MTC 44-2 cells, which express the Ca^{2+} receptor *(9)*, were a generous gift of Armen H. Tashjian. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 15% horse serum and 1 mmol/L L-glutamine, in 75- or 150-cm^2 flasks at 37°C

in a humidified atmosphere of 95% O_2, 5% CO_2. All media and components were obtained from Gibco-BRL (Gaithersburg, MD). The culture medium was changed every 2–3 d, and the cells were passaged by trypsinization in 1 mmol/L EDTA containing 0.25% trypsin once a week. Cells to be used for calcitonin secretion experiments were trypsinized and replated in 1.0-cm^2 48-well plates at a density of approx 100,000 cells/well 2–5 d prior to use. Calcitonin secretion experiments were performed in DMEM/F-12 medium, supplemented with 5% rat serum and L-glutamine (1 mmol/L). On the day of the experiment, the cells were washed twice with Dulbecco's phosphate-buffered saline. They were then incubated on an oscillating shaker (80 cycles/min) in 1 mL of experimental medium for 4 h at 37°C in a humidified atmosphere of 95% O_2, 5% CO_2. To assess the effects of Ca^{2+} receptor activation on calcitonin secretion by MTC 44-2 cells, the experimental media contained varying concentrations of Ca^{2+} and NPS R-467. The medium was removed at the end of the incubation and directly assayed for calcitonin.

In Vivo Studies in Rats

These studies were performed in male Sprague-Dawley rats (Harlan Sprague Dawley) initially weighing 250–275 g. They were provided with unrestricted access to a commercial rodent chow (Purina 5001) containing 1.0% Ca, 0.7% P, and tap water. Most rats had blood sampling and infusion catheters implanted chronically in the abdominal aorta and inferior vena cava, respectively, at least 3 d prior to study *(36)*. To investigate the effects of chronic renal insufficiency on plasma levels of calcitonin, one group of rats was subjected to a one-stage 5/6 nephrectomy (Nx) procedure, as described previously *(37)*. Control animals received a sham operation. The sham-operated and 5/6 Nx rats were fed a semisynthetic diet containing 0.6% Ca and 1.2% P (Teklad, Madison, WI) for 6 w before a blood sample was collected. All experimental procedures were approved by the Institutional Animal Care and Use Committee of NPS Pharmaceuticals.

Effects of Hypocalcemia or Thyroparathyroidectomy

The effects on calcitonin levels of acute induction of hypocalcemia were studied in five chronically catheterized, normal conscious rats. Following the collection of a basal blood sample (1.0 mL), 0.2–0.3 mL of a 100 mmol/L solution of EGTA, pH 7.4, was infused iv over about 10 min to induce a sustained decrease in plasma Ca^{2+} levels. A second blood sample was collected about 15 min after the start of the EGTA infusion. The catheter was flushed with 1.0 mL saline after each sample was collected. Plasma Ca^{2+} levels were measured immediately and plasma was stored at –20°C for subsequent calcitonin assay.

A blood sample (1.0 mL) was collected from eight catheterized, normal conscious rats. Then, each rat was anesthetized with a combination of ketamine (90 mg/kg) and xylazine (7 mg/kg) injected intramuscularly and thyroparathyroidectomized. The rats were allowed to recover from the anesthesia, and additional blood samples were collected at 4 and 24 h after surgery. The catheter was flushed with 1.0 mL saline after each sample was collected.

Effects of Hypercalcemia or NPS R-467

Normal rats were infused iv (0.167 mL/min) with calcium gluconate at rates of 5, 10, 15, 22.5, and 30 μmol/min for 2 min. A control group was infused with saline. Blood samples for Ca^{2+} and calcitonin assays were collected immediately before, and at 2, 5, 10 and 15 min after the start of the infusion. Other similar rats received by gavage (1 mL/200 g) NPS R-467 (1–750 μmol/kg) or the vehicle, which was a 15% aqueous solution of 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals, Natick, MA). Blood samples (0.8 mL) for analyses of the plasma levels of calcitonin, PTH, and Ca^{2+} were collected immediately before, and at 15, 30, 45, 60, 90, and 120 min after dosing. To prevent excessive blood loss associated with repeated sampling, the red cell pellet from each blood sample was resuspended in an equal volume of normal rat plasma and reinjected.

In Vivo Studies in Mice

Normal adult Balb/c mice (Harlan Sprague Dawley) fed Teklad 8640 rodent chow (1.3% Ca, 0.95% P) received an ip injection of 0.9% saline or Ca (500 μmol/kg). The mice were anesthetized with isoflurane, and a blood sample for calcitonin assay was collected by cardiac puncture 10 min after the injection.

Other Analyses

Ionized calcium (Ca^{2+}) levels were measured in MTC 44-2 cell culture media and in whole blood immediately after collection using a model 634 Ca^{2+} analyzer (Ciba Corning Diagnostics, Medford, MA). BUN and phosphate levels were measured using a multichannel analyzer (Monarch 1000; Instrumentation Laboratory, Lexington, MA). PTH was measured using a rat PTH-(1-34) IRMA kit (Immutopics, San Clemente, CA).

Statistical Analysis

Values are expressed as mean ± SE. A *t*-test or when variances were unequal the Mann-Whitney U-test was used to determine the significance of differences between conscious and anesthetized rats and between sham-operated and 5/6 Nx rats. Analysis of variance followed by Dunnett's test was employed when multiple comparisons were required; $p < 0.05$ was used to indicate a significant difference. EC_{50} and ED_{50} values in dose–response studies were determined using the Levenberg-Marquardt algorithm (KaleidaGraph, Abelbeck Software).

Acknowledgments

Parts of this work were presented at the 79th Annual Meeting of the Endocrine Society, Minneapolis, MN, June 11–14, 1997.

References

1. Broadus, A. E. (1996). In: *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism,* 3rd Ed. Favus, M. J. (ed.). Lippincott-Raven: Philadelphia, pp., 57–63.
2. Deftos, L. J. (1996). In: *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism,* 3rd Ed. Favus, M. J. (ed.). Lippincott-Raven: Philadelphia, pp., 82-87.,
3. Becker, K. L., Nylén, E. S., Cohen, R., and Snider, R. H., Jr. (1996). In: *Principles of Bone Biology,* Bilezekian, J. P., Raisz, L. G., and Rodan, G. A. (eds.). Academic: San Diego, pp. 471–494.
4. Cooper, C. W., Hirsch, P. F., and Munson, P. L. (1970). *Endocrinology* **86,** 406–415.
5. Yamamoto, M., Seedor, J. G., Rodan, G. A., and Balena, R. (1995). *Endocrinology* **136,** 788–795.
6. Austin, L. A. and Heath, H., III. (1981). *N. Engl. J. Med.* **304,** 269–278.
7. Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., et al. (1993). *Nature* **366,** 575–580.
8. Garrett, J. E., Capuano, I. V., Hammerland, L. G., Hung, B. C. P., Brown, E. M., Hebert, S. C., et al. (1995). *J. Biol. Chem.* **270,** 12,919–12,925.
9. Garrett, J. E., Tamir, H., Kifor, O., Simin, R. T., Rogers, K. V., Mithal, A., et al. (1995). *Endocrinology* **136,** 5202–5211.
10. Nemeth, E. F. (1996). In: *Principles of Bone Biology,* Bilezekian, J. P., Raisz, L. G., and Rodan, G. A. (eds.). Academic: San Diego, pp. 1019–1035.
11. Nemeth, E. F., Steffey, M. E., Hammerland, L. G., Hung, B. C. P., Van Wagenen, B. C., Delmar, E. G., et al. (1998). *Proc. Natl. Acad. Sci. USA* **95,** 4040–4045.
12. Fox, J., Hadfield, S., Petty, B. A., and Nemeth, E. F. (1993). *J. Bone Miner. Res.* **8(Suppl. 1),** S181 (Abstract).
13. Fox, J., Hadfield, S., Petty, B. A., Conklin, R. L., and Nemeth, E. F. (1993). *J. Am. Soc. Nephrol.* **4,** 719 (Abstract).
14. Raue, F., Deutschle, I., Küntzel, C., and Ziegler, R. (1984). *Endocrinology* **115,** 2362–2367.
15. Feinstein, R. E., Bucht, E., Grimelius, L., Iwarsson, K., Rönnbäck, C., and Sjöberg, H. -E. (1989). *Acta Endocrinol. (Copenh.)* **120,** 210–214.
16. Fox, J. (1988). *Am. J. Physiol.* **255,** E702–E707.
17. Deftos, L. J., Roos, B. A., Bronzert, D., and Parthemore, J. G., (1975). *J. Clin. Endocrinol. Metab.* **40,** 409–412.
18. Body, J. -J. and Heath H., III. (1983). *J. Clin. Endocrinol. Metab.* **57,** 897–903.
19. Antonsen, J. E., Sherrard, D. J., and Andress, D. L. (1998). *Kidney Int.* **53,** 223–227.
20. Rehli, M., Luger, K., Beier, W., and Falk, W. (1996). *Biochem. Biophys. Res. Commun.* **226,** 420–425.
21. Kovacs, C. S., Ho-Pao, C. L., Hunzelman, J. L., Lanske, B., Fox, J., Seidman, J. G., et al. (1998). *J. Clin. Invest.* **101,** 2812–2820.
22. Burford, H. J., Ontjes, D. A., Cooper, C. W., Parlow, A. F., and Hirsch, P. F. (1975). *Endocrinology* **96,** 340–348.
23. Raulais, D., Hagaman, J., Ontjes, D. A., Lundblad, R. L., and Kingdon H. S. (1976). *Eur. J. Biochem.* **64,** 607–611.
24. Heath, H. III., Body, J. -J., and Fox, J. (1984). *Biomed. Pharmacother.* **38,** 214–245.
25. Okumura, K., Saito, Y., Yasuhara, M., and Hori, R. (1984). *J. Pharm. Dynamics* **7,** 917–922.
26. Schultz, V. L., Boass, A., Garner, S. C., and Toverud, S. U. (1995). *J. Bone Miner. Res.* **10,** 1298–1302.
27. Wixson, S. K., White, W. J., Hughes, H. C., Jr., Lang, C. M., and Marshall W. K. (1987). *Lab. Anim. Sci.* **37,** 736–742.
28. Ardaillou, R. (1975). *Nephron* **15,** 250–260.
29. Mithal, A., Kifor, O., Kifor, I., Vassilev, P., Butters, R., Krapcho, K., et al. (1995). *Endocrinology* **136,** 3087–3092.
30. Nemeth, E. F. (1990). *Cell Calcium* **11,** 323–327.
31. Kifor, O., Diaz, R., Butters, R., and Brown, E. M. (1997). *J. Bone Miner. Res.* **12,** 715–725.
32. Schultz, V. L., Garner, S. C., Lavigne, J. R., and Toverud, S. U. (1994). *Bone Miner.* **27,** 121–132.
33. Rucinski, B., Mann, G. N., and Epstein, S. (1995). *Calcif. Tissue Int.* **56,** 83–87.
34. Reichlin, M. (1980). *Methods Enzymol.* **70,** 159–165.
35. Hunter, W. M. and Budd, P. S. (1981). *J. Immunol. Methods* **45,** 255–273.
36. Fox, J. (1990). *Horm. Metab. Res.* **22,** 278–282.
37. Rogers, K. V. and Fox, J. (1995). *Endocrine* **3,** 769-774.