Investigations of the Molecular Changes Occurring in Secondary Hyperparathyroidism in a Uremic Rat Model

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ABSTRACT

Chromogranin-A (CgA) and parathyroid hormone (PTH) are the two major secretory products of the parathyroid gland. CgA, the principal member of the granin family of acidic glycoproteins, is widely distributed in endocrine and nervous tissue. While CgA is believed to be important for cell secretory activity and to function in both an intracellular and an extracellular manner, its precise function(s) remain to be fully elucidated. In order to gain a better understanding of the regulation of CgA gene expression *in vivo*, the effects of the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) were investigated on parathyroid gland CgA mRNA in the rat. Parathyroid CgA mRNA levels increased in a dose-dependent manner in response to injections of $1,25(OH)_2D_3$ whereas PTH mRNA levels decreased. Notably, under these conditions, CgA mRNA levels were less sensitive to $1,25(OH)_2D_3$ than PTH mRNA levels. Having established *in vivo* regulation of CgA by $1,25(OH)_2D_3$, the focus was then to explore the potential role of altered expression of CgA in the pathophysiology of secondary hyperparathyroidism (2°HPT) using the uremic rat model. Five-sixth (5/6) nephrectomized rats maintained on a normal diet had normal levels of serum calcium and phosphate but reduced levels of $1,25(OH)_2D_3$. In these uremic rat parathyroid glands whereas PTH mRNA levels were four-fold higher, CgA mRNA levels were 50% lower that those in the sham-operated rats. Therefore, in the presence of normocalcemia and reduced circulating $1,25(OH)_2D_3$ levels the parathyroid CgA mRNA level is altered, but in the opposite direction to that of PTH mRNA. In uremic rats maintained on a high phosphorus diet, 5/6 nephrectomy resulted in decreased serum calcium and $1,25(OH)_2D_3$ levels. In contrast to uremic rats on a normal diet, these animals had elevated parathyroid CgA and PTH mRNA levels. Thus, these studies show that *in vivo*, increased serum $1,25(OH)_2D_3$ concentrations stimulate, and decreased serum levels inhibit parathyroid CgA mRNA expression. However, this latter effect depends upon the calcium status of the animal. These studies have also provided indirect evidence that CgA regulates PTH secretion in a predominantly extracellular manner.

RÉSUMÉ

La chromogranine A (CgA) et l'hormone parathyroïdienne (PTH) sont les deux produits majoritaires sécrétés par les glandes parathyroïdiennes. La CgA, principal membre d'une famille de glycoprotéines acides nommées "granines", est largement exprimées dans les tissus endocriniens et neuroendocriniens. Malgré que les fonctions spécifiques de cette protéine ne soient pas encore établies, il a été suggéré que la CgA pourrait jouer un rôle dans les processus de sécrétion cellulaire. Afin de mieux comprendre la régulation de la CgA *in vivo*, nous avons mesuré le niveau d'expression de l'ARNm de la CgA dans les glandes parathyroïdiennes, suite à l'administration du métabolite actif de la vitamin D_3, la 1,25-dihydroxyvitamine D_3 ($1,25(OH)_2D_3$) à des rats normaux. Nous avons observé que les injections de $1,25(OH)_2D_3$ augmentaient l'expression de l'ARNm de la CgA mais diminuaient celle de l'ARNm du PTH, de façon dose-dépendante. Cependant, les variations d'expression des messagers dû au traitement par la $1,25(OH)_2D_3$ sont plus importantes dans le cas du PTH que de la CgA. Après avoir démontré *in vivo*, la régulation de l'ARNm de la CgA par la $1,25(OH)_2D_3$ dans le rat normal, nous avons étudié le rôle potentiel des modifications de concentration de la CgA dans la pathophysiologie de l'hyperparathyroïdisme secondaire en utilisant un modèle de rats rendus urémiques par nephrectomie. Chez les rats néphrectomisés et maintenus dans une diète normale, nous avons observé une diminution de la concentration plasmatique de la $1,25(OH)_2D_3$ alors que celles du calcium et du phosphore restaient inchangées. Parallèlement à la diminution de la concentration plasmatique de la $1,25(OH)_2D_3$ chez les animaux urémiques, une diminution de 50% de l'expression du messager de la CgA dans les glandes parathyroïdiennes a aussi été mise en évidence. Par contre, ces même animaux urémiques présentaient une

augmentation de 4 fois au-dessus de la normale de l'expression du messager du PTH. Dans un autre groupe de rats néphrectomisés mais cette fois maintenus dans une diète élevée en phosphore, nous avons noté une dimunation des concentrations plasmatiques de calcium et de $1,25(OH)_2D_3$ mais une augmentation de l'expression parathyroïdïenne de l'ARNm de la CgA et du PTH étaient augmentées. L'ensemble de ces études démontrent donc que l'expression de l'ARNm de la CgA parathyroïdienne est stimulée en présence de concentrations plasmatiques élevées de $1,25(OH)_2D_3$ tandis qu'elle est inhibée lorsque les concentrations plasmatiques de $1,25(OH)_2D_3$ sont faibles. Cependant, ce dernier effet est dépendant de la concentration calcique plasmatique. Ces études démontrent aussi que la CgA affecte la sécrétion du PTH d'une façon plus tôt extracellulaire.

PREFACE

During my MSc studies at the Calcium Research Laboratory I have been involved in several projects and have made contributions to the following manuscripts and abstracts:

A) Reviewed Manuscripts

1. Janicic, N., Soliman, E., Pausova, Z., Seldin, M.F., Szpirer, C. and G.N. Hendy. Mapping of the Ca^{2+}-sensing receptor gene to human chromosome 3q13.3-21 by fluorescence in situ hybridization, and localization to rat chromosome 11 and mouse chromosome 16. *Mammalian Genome*. 6:798-801. 1995.

2. Pausova, Z., Soliman, E., Amizuka, N., Janicic, N., Konrad, E.M., Arnold, A., Goltzman, D. and G.N. Hendy. Role of RET proto-oncogene in sporadic hyperparathyroidism and in hyperparathyroidism of multiple endocrine neoplasia type 2. *J. Clin. Endocrinol. Metab.* In Press. 1996.

B) Manuscripts Submitted

1. Soliman, E., Fox, J. and Hendy, G.N. *In vivo* regulation of chromogranin A mRNA in the parathyroid by 1,25-dihydroxyvitamin D. Submitted 1996.

C) Abstracts

1. Soliman, E., Bourdon, J., Fox, J. and G.N. Hendy. Expression of parathyroid hormone mRNA in normal and uremic rats. *J. Bone Min. Res.* 8, 435, S225, 1993.

2. Sohi, J., Soliman, E., Goltzman, D. and G.N. Hendy. Proliferative role of c-myc in parathyroid (PT-r) cells investigated using antisense oligomers. *J. Bone Min. Res.* 8, 436, S225, 1993.

3. Soliman, E., Fox, J. and G.N. Hendy. Parathyroid chromogranin A mRNA is regulated by 1,25-dihydroxyvitamin D_3 *in vivo*: reduced expression in the uremic rat. *J. Bone Min.Res.* 9, A476, S222, 1994.

4. Pausova, Z., Janicic, N., Konrad, E., Soliman, E., Goltzman, D. and G.N. Hendy. Analysis of the RET proto-oncogene in sporadic parathyroid tumors. *J. Bone Min. Res.* 9, 122, S151, 1994

5. Pausova, Z., Janicic, N., Konrad, E., Soliman, E., Hershenfield, D., Goltzman, D. and G.N. Hendy. Absence of RET proto-oncogene abnormalities in sporadic parathyroid tumors. *Amer. J. Hum. Genet.* 55s3, A66, Abs 359, 1994.

6. Janicic, N., Soliman, E., Pausova, Z., Seldin, M.F., Szpirer, C. and G.N. Hendy. Mapping of the calcium-sensing receptor gene (CASR) to human chromosome 3q13.3-21 by fluorescence in situ hybridization, and localization to rat chromosome 11 and mouse chromosome 16. *J. Bone Min. Res.* 10, M501, S377, 1995.

7. Pausova, Z., Soliman, E., Amizuka, N., Janicic, N., Konrad, E.M., Arnold, A., Goltzman, D. and G.N. Hendy. Expression of the Ret proto-oncogene in hyperparathyroid tissues: implications for the pathogenesis of the parathyroid disease in MEN2A. *J. Bone Min. Res.* 10, P249, S191, 1995.

8. Soliman, E., Fox, J., Lin, J., Du, X. and G.N. Hendy. Parathyroid chromogranin A (CgA) acts intracellularly to modulate parathyroid hormone (PTH) secretion *in vivo*. 10th International Congress of Endocrinology, 1996.

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ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-dihydroxycholecalciferol
$25(OH)D_3$	25-hydroxy-vitamin D_3
2°HPT	Secondary hyperparathyroidism
9-*cis*-RA	9-*cis*-retinoic acid
ACTH	adrenocorticotropin
BoPCaR1	Bovine parathyoid calcium sensing receptor
BSA	Bovine serum albumin
Ca^{2+}	Ionized calcium
Calcitriol	1,25-dihydroxycholecalciferol
cAMP	3',5'-cyclic adenosine monophosphate
CaR	Calcium sensing receptor
CgA	Chromogranin A
CgB	Chromogranin B
CRE	Cyclic AMP-responsive element
CREB	Cyclic AMP response element-binding protein
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FMEN-I	Familial multiple endocrine neoplasia type 1
g	Gram
G-protein	Guanine nucleotide binding protein
H_2O	Water
LiCl	Lithium chloride
M	Molar
mL	Millilitre
mM	Millimolar
mRNA	Messenger RNA
$NaPO_4$	Sodium phosphate
POMC	Pro-opiomelanocortin
preproPTH	Preproparathyroid hormone
proPTH	Proparathyroid hormone
PTH	Parathyroid hormone
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Tris-HCL	Tris(hydroxymethyl)aminomethane-hydrochloride
VDR	Vitamin D_3 receptor
VDRE	Vitamin D_3 receptor element
x *g*	Relative centrifugal force
µl	Microlitre

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PART 1: INTRODUCTION

A. CALCIUM HOMEOSTASIS

Maintenance of the circulating concentration of ionized calcium within a very narrow range (1-1.3 mM) is essential for the normal functioning of numerous biochemical processes within the body. The two major hormones responsible for calcium homeostasis are parathyroid hormone (PTH) and the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or calcitriol. PTH is an 84 amino acid polypeptide synthesized in the parathyroid chief cell and its release into the circulation represents the primary response to a hypocalcemic stimulus. The mature form of this protein is derived from a larger molecule, prepro-PTH, encoded by the PTH gene (Kemper et al., 1974). In the endoplasmic reticulum, the signal peptide or "pre" sequence is cleaved by signal peptidases and recent evidence (Hendy et al., 1995) strongly implicates furin as the prohormone convertase responsible for the processing of proPTH to PTH. At the molecular level, this hormone mediates its effects by binding the PTH/PTHrP receptor (Juppner et al., 1991), a G protein-linked cell surface receptor that acts through second messengers such as cAMP (Chase et al., 1967) and free intracellular calcium via phosphotidylinositol turnover (Meltzer et al., 1982; Yamaguchi et al., 1987). This receptor also binds PTH-related peptide, originally described as the humoral factor responsible for malignancy-associated hypercalcemia. Binding and subsequent activation of the receptor requires only the first 34 amino acids of the PTH molecule, whose principal action is to increase extracellular calcium by enhancing bone resorption, by acting on the kidney to promote reabsorption of calcium in the distal tubules and by stimulating the synthesis of $1,25(OH)_2D_3$ via activation of the enzyme 25-hydroxyvitamin D 1-hydroxylase in the proximal convoluted tubules (Garabedian et al., 1972). $1,25(OH)_2D_3$, in turn, acts on the intestine to increase the rate of absorption of calcium, while also having a permissive effect of PTH on bone. In addition, it inhibits parathyroid gland activity namely by decreasing the production of PTH, thereby completing a classical endocrine negative feedback loop designed to prevent major fluctuations in the concentration of extracellular calcium.

B. REGULATION OF THE PARATHYROID GLAND BY CALCIUM

The major physiological regulators of parathyroid gland activity are the extracellular ionized calcium concentration and $1,25(OH)_2D_3$. *In vivo* (Mayer et al., 1978) and *in vitro* (Brown et al., 1977) experiments have shown that the relationship between PTH secretion and extracellular calcium concentration can be represented graphically by an inverse sigmoidal line characterized by a steep slope. This last feature derives from the exquisite sensitivity by which the glands respond to slight alterations in serum ionized calcium. Our understanding of the molecular mechanisms underlying calcium sensing by the parathyroid gland as well as other calcium responsive organs has improved considerably since the cloning of a calcium receptor from bovine parathyroid (BoPCaR1) (Brown et al., 1993). The human calcium-sensing receptor gene has been mapped to Chromosome (Chr) 3, localized to rat Chr 11 and mouse Chr 16. Belonging to the superfamily of seven membrane-spanning G protein-coupled receptors, BoPCaR functions in part, via activation of the phosphotidylinositol pathway (Brown et al., 1993). Certain hereditary diseases characterized by reduced tissue reponsiveness to concentration changes of extracellular calcium, including familial hypocalciuric hypercalemia and neonatal severe hyperparathyroidism, have been attributed to inactivating mutations in the gene of the human calcium-sensing receptor (Pollak et al., 1993, Janicic et al., 1995).

Parathyroid glands have a tremendous adaptive response due in large part to the various potential levels of regulation involved in biosynthesis and secretion of PTH. Acute control of PTH secretion is dependent on the rate of release of preformed hormone and extent of intracellular degradation of these same preexisting stores (Habener et al., 1975; Hanley et al., 1978; Mayer et al., 1979). For example, under normal or high ambient calcium concentrations, a large amount of PTH is broken down in the parathyroid cell yielding inactive mid-region or carboxy-terminal fragments to be secreted (Hanley et al., 1978). These circulating moieties however, also originate mainly from hepatic and to a lesser extent, renal cleavage of the intact hormone (Hruska et al., 1977; D'Amour et al., 1979; Canterbury et al., 1975). Inactive fragments comprising mostly carboxy-terminal fragments account for approximately 70-95% of the total circulating immunoreactive hormone (Segre, 1993) and because these fragments are cleared solely by the kidney, their concentration increases drastically in renal failure (Martin et al., 1979). On the other hand,

clearance of amino-terminal fragments seem to be unaffected, probably due to increased metabolism by peripheral organs. Under such conditions therefore, an amino-terminal or 'intact' PTH radioimmunoassay is necessary to correctly assess parathyroid status. In response to hypocalcemic conditions, less degradation takes place in the parathyroid gland and therefore more of the biologically active intact PTH (1-84) is secreted into the circulation (Mayer et al., 1979).

Chronic changes in serum calcium, on the other hand, involves changes at the level of PTH gene transcription. *In vitro* experiments using primary cultured bovine parathyroid cells demonstrate that whereas low medium calcium concentrations (0.4 mM) either slightly increase steady state preproPTH mRNA levels (Brookman et al., 1986) or have no effect (Russell et al., 1983), a high medium calcium concentration (2.5 mM) causes a substantial reversible reduction in PTH mRNA, the first significant change being observed as early as 16 hours (Russell et al., 1983). The effect of calcium on the rate of PTH transcription seems to be a direct one (Russell and Sherwood, 1987). Assuming that the above data can be extrapolated to the *in vivo* setting, it would mean that under low or normal calcium concentrations, PTH synthesis occurs at a near maximal rate and that post-translational events such as the rates of secretion and degradation are the major determinants of PTH release. Conversely, chronic exposure to high ambient calcium levels results not only in changes in the rates of secretion and degradation, but also to decreased synthesis. A major obstacle involved with using primary cultures of bovine parathyroid cells is that they progressively become less responsive to changes in extracellular calcium. Mithal and associates (1995) demonstrated that the expression of the calcium sensing receptor diminishes with time, however this decrease is retarded if the cells are grown in a low serum concentration. This is consistent if we consider that markers of the differentiated state, such as the calcium sensing receptor, are decreased under conditions of stimulated cellular growth. While the authors suggest that a decreased calcium receptor mRNA could contribute to the loss of responsiveness to calcium observed in such culture systems, further work must be done in this area to explore other potential factors that could contribute to phenotypic differences in cultured parathyroid cells as compared to *in vivo*.

In contrast to the above *in vitro* studies, *in vivo* experiments demonstrated that rats rendered hypocalcemic following injection of phosphorus have higher levels of PTH mRNA as early as 1 hour (Naveh-Many et al., 1989). These same studies however, found no effect

following deviation of serum calcium demonstrating the presence of a "non-suppressible" component of PTH gene expression. Another group (Yamamoto et al., 1989) also observed an increase in hypocalcemic rats, however these changes occurred at 48 but not 24 hours after the infusion. They were also able to show a slight suppression in steady state PTH mRNA levels under hypercalcemic conditions. These conflicting results in response and required time course of action between the two groups may be explained by differences in endogenous calcitriol levels which were not quoted. Thus, unlike *in vitro* studies, these experiments indicate that PTH gene expression is normally kept at a relatively low level but can be increased sharply upon a hypocalcemic stimulus.

C. REGULATION OF PARATHYROID FUNCTION BY $1,25(OH)_2D_3$

$1,25(OH)_2D_3$, the other principal regulator of parathyroid gland synthetic and secretory activity is a sterol that contributes to mineral homeostasis by mediating calcemic effects on its classic target organs; the intestine, bone and kidney. $1,25(OH)_2D_3$ mediates its genomic effects by binding to the nuclear vitamin D receptor (VDR) (Haussler and Norman, 1969), which belongs to the steroid, thyroid, and retinoic acid receptor superfamily (Evans et al., 1988). These receptors are ligand induced transcription factors composed of several highly conserved domains including a DNA binding zinc finger domain located at the amino-terminus and a ligand binding domain located at the carboxy-terminus (McDonnell et al., 1989). The complex that subsequently forms regulates gene transcription by interacting directly with a specific cis-acting DNA sequence on the target gene also known as a vitamin D-responsive element (VDRE) (Kerner et al., 1989). Indeed, the identification of specific receptors for $1,25(OH)_2D_3$ in parathyroid cells (Brumbaugh et al., 1975) as well as an up-stream negative response element in the PTH gene (Demay et al., 1992) implicates this sterol as an important physiological regulator of parathyroid gland function.

The VDRE consensus sequence is composed of two direct repeats of six nucleotides having the motif PurG(G/T)TCA, separated by three nucleotide base pairs (De Luca, 1991). Efficient binding of the VDR to DNA is dependent on the receptor forming a transcriptionally active heterodimer with another nuclear accessory factor (Liao et al, 1990; Sone et al., 1991), the nuclear retinoid X receptor (RXR) (Yu et al., 1991) which has 9-*cis*-

retinoic acid (9-*cis*-RA) as its natural ligand (Heyman et al., 1992). In this regard, evidence implicating retinoids in regulation of PTH expression comes from a study demonstrating inhibition of synthesis and secretion in cultured bovine parathyroid cells by 9-*cis*-RA independent of $1,25(OH)_2D_3$ (MacDonald et al., 1994). Moreover, the authors were able to demonstrate an additive inhibitory effect when both compounds were added to the culture medium.

$1,25(OH)_2D_3$ reversibly suppresses steady-state preproPTH mRNA levels by 50% at 48 hours in primary cultures of bovine parathyroid cells (Silver et al., 1985), some groups observing a significant decrease as early as 6 hours (Russell et al., 1986). This action is mediated directly at the level of gene transcription rather than mRNA stability (Russell et al., 1986,1990; Mouland and Hendy, 1991; Silver et al., 1986). Furthermore, the lack of any short-term effects by $1,25(OH)_2D_3$ on secretion (Cantley et al., 1987) tends to rule out modulation of preformed PTH stores or secretion and indicates that the decreases in mRNA levels are probably solely responsible for the eventual reductions in production and secretion. In normal rats, pharmacological doses of $1,25(OH)_2D_3$ that do not cause hypercalcemia suppress serum immunoreactive PTH levels as early as four hours (Chertow et al., 1975) as well as prepro PTH mRNA levels in a dose-dependent manner (Silver et al., 1986). In contrast, PTH mRNA levels are increased in a vitamin D-deficient, normocalcemic state caused either by dietary means (Hendy et al.,1989; Naveh-Many and Silver, 1990) or induction of experimental uremia (Shvil et al., 1990). The combination of hypocalcemia and low vitamin D levels has an additive stimulatory effect on PTH gene expression as would be expected, suggesting a different regulatory path for the mechanism of action of these two factors.

Other studies however, point to a more complicated means of regulation. Depending on the circumstance, it appears that either $1,25(OH)_2D_3$ or calcium can have a dominant role in regulating the PTH gene. For example, in rats initially injected with phosphorus (a calcium lowering agent), calcitriol administration leads to normocalcemia but reduced levels of PTH mRNA (Naveh-Many et al., 1989) and circulating protein (Chertow et al., 1975). Conversely, rats fed a low-calcium diet, resulting in hypocalcemia but elevated $1,25(OH)_2D_3$ concentrations, have increased PTH mRNA levels indicating an overriding effect of calcium on the sterol under those particular conditions (Naveh-Many and Silver, 1990).

D. PATHOGENESIS OF SECONDARY HYPERPARATHYROIDISM

Secondary hyperparathyroidism is a disease invariably associated with chronic renal failure. This disease is characterized by enlargement and disordered regulation of the parathyroid glands leading to hypersecretion of PTH and consequently bone disease including osteitis fibrosa and osteomalacia (Goodman et al., 1993). In a more severe form of the disease, termed tertiary hyperparathyroidism, the parathyroid glands are no longer responsive and hence are considered to be in an autonomous state of secretion.

In contrast to primary hyperparathyroidism where an intrinsic glandular defect gives rise to excessive PTH secretion, 2°HPT is the result of an adaptive process. The abnormal changes observed in serum chemistry caused by renal insufficiency play a role in altering the normal regulation and secretion of PTH. The exact etiology of 2°HPT is still unknown, however, because calcium is the major regulator of parathyroid gland function and hypersecretion of PTH is considered to be an adaptive response to protect against low circulating calcium levels, hypocalcemia has always been regarded as an important factor in the development of 2°HPT. Earlier studies (Slatopolsky et al., 1972; Bricker et al., 1972) gave rise to the "trade-off hypothesis" which proposed that phosphate retention manifested by a transient reduction in the renal filtered load, leads to a corresponding decrease in plasma calcium. As a result, PTH hypersecretion is "traded-off" in order to maintain mineral homeostasis.

Over the years, it has become clear that hypocalcemia *per se* may not be the primary factor giving rise to 2°HPT. For example, in contrast to severe chronic renal failure, Pitts and co-workers (1988) discovered normal calcium levels in patients with mild or moderate renal failure. It should be noted however, that all three groups were found to be $1,25(OH)_2D_3$ deficient. Furthermore, a study by Lopez-Hilker et al. (1986) demonstrated that hypocalcemia is not essential for the development of 2°HPT. In this study, dogs with chronic renal failure were fed high calcium diets in order to prevent hypocalcemia. Despite this treatment, 2°HPT developed in the presence of reduced levels of calcitriol. Administration of $1,25(OH)_2D_3$ in the same model prevented an increase in serum levels of PTH suggesting that calcitriol deficiency contributes to abnormal parathyroid gland function independent of a reduction in serum calcium levels.

Besides hypocalcemia and calcitriol deficiency, other factors are believed to play a

role in the pathogenesis of 2°HPT. These include phosphorus retention (Slatopolsky et al., 1972), renal and skeletal resistance to the calcemic action of PTH (Sommerville et al., 1978), and alterations to normal calcium-mediated PTH secretion (Brown et al., 1982). Because of the complexity of this disease, the relative contribution of these various elements towards the development of parathyroid hyperplasia remains unclear. Furthermore, these factors are extensively interrelated and not mutually exclusive, making it difficult to elucidate the effect of any one factor alone in the pathogenesis of this disease.

Although it is presently unclear, increased glandular size and secretion is believed to result either from an increase in number of cells (hyperplasia) and/or the size of the cells (hypertrophy). Clinically, parathyroid gland hyperplasia is often associated with conditions of prolonged stimulation such as chronic hypocalcemia or reduced levels of $1,25(OH)_2D_3$. As mentioned already, these factors, among others, are believed to be major contributors to the hyperparathyroidism observed in chronic renal failure.

In vitro studies focusing on the effects of low calcium on parathyroid cell proliferation have been inconsistent. Some groups have observed an increase (Roth and Raisz, 1964; Lee and Roth, 1975, Brandi et al., 1986) whereas others found no change in cellular proliferation (Kremer et al., 1989; Leboff et al., 1983). *In vivo*, the increased gland size in rats fed a low calcium diet was attributed solely to cell hypertrophy rather than hyperplasia implying an increase in PTH secretion per cell (Wernerson et al., 1990). Likewise, Szabo et al. (1989) found that the rate of cellular proliferation, as assessed by ^{3}H-thymidine incorporation into parathyroid glands of rats fed a similar diet, were not different from control despite an increase in glandular weight. Although the rats in the above two studies were hypocalcemic, an effect by $1,25(OH)_2D_3$ cannot be excluded since endogenous levels of this sterol are greatly increased in these dietary models. It is conceivable therefore that such an increase counteracted the effects of hypocalcemia on parathyroid cell proliferation. A recent study however has shed some light upon this issue. Naveh-Many and associates (1995) found that a low calcium diet fed to rats leads to increased PTH gene expression as well as increased parathyroid cell proliferation in the presence of reduced calcium but elevated levels of $1,25(OH)_2D_3$. This indicates an overriding effect of calcium on growth. In contrast, no parathyroid cell proliferation is detected in rats fed a low phosphorus diet which led to elevated serum concentrations of calcium and $1,25(OH)_2D_3$ but reduced phosphate and PTH mRNA levels.

The effect of $1,25(OH)_2D_3$ on cellular proliferation is more apparent. Considerable evidence exists indicating that calcitriol suppresses cell growth *in vitro* (Nygren et al., 1988) at least in part by inhibiting expression of *c-myc* proto-oncogene (Kremer et al., 1989). $1,25(OH)_2D_3$ has also been shown to reduce proliferation of parathyroid cells *in vivo* (Szabo et al., 1989). Moreover, vitamin D-deficient, normocalcemic dogs experience parathyroid gland hyperplasia and elevated PTH levels (Hendy et al., 1989) whereas administration of $1,25(OH)_2D_3$ prevents parathyroid gland hyperplasia from developing in nephrectomized dogs (Lopez-Hilker et al., 1986). These studies are consistent with $1,25(OH)_2D_3$ being not only a physiological regulator of parathryoid sythesis and secretion but also an important regulator of parathyroid cell proliferation.

Finally, in rats fed a diet deficient in both calcium and vitamin D, the increase in PTH gene expression (10-fold normal) was attributed mostly to an increase in PTH mRNA per cell rather than cell number since the latter was only 1.7 fold higher than normal (Naveh-Many and Silver, 1990).

The fact that 2°HPT is a multiglandular disease may suggest a polyclonal origin unlike 1°HPT that is monoclonal (Arnold et al., 1995). Initial assessments of allelic loss at 11q13, the multiple endocrine neopasia type 1 locus (Larsson et al., 1988) which is found in 3% of parathyroid adenomas in 1°HPT, found that only 2 of 12 patients with hyperparathyroidism secondary to chronic renal failure had tumour clonality (Falchetti et al., 1993). Recent data, however, does not support the occurrence of generalized enlargement of cells in this disease. Arnold et al. (1995) have demonstrated monoclonality of parathyroid neoplasms by means of X-chromosome inactivation analysis in at least 7 of 11 patients with uremic refractory 2°HPT. One patient had tumour-specific monoclonal loss of heterozygosity on the X-chromosome while none had allelic loss at 11q13. Interestingly, it appears that two glands from the same patient can have different clonal origins.

i. Role of Phosphate

Phosphate retention has long been regarded as one of the primary abnormalities associated with the onset of hyperparathyroidism secondary to chronic renal failure (Bricker et al., 1969; Slatopolsky et al. 1972, 1973; Kaplan et al., 1979). The biochemical changes which ensue are believed to be part of a compensatory mechanism whose purpose is to

maintain phosphate homeostasis which is compromised due to the impaired ability of the kidney to excrete phosphorus.

Early studies have revealed that 2°HPT in dogs with experimental uremia can be improved (Kaplan et al., 1979) or even prevented (Slatopolsky et al., 1972) by proportional dietary phosphorus restriction. While dietary phosphorus restriction and phosphate-binding agents are used clinically in the management of 2°HPT (Goodman et al., 1993), the most severe form of experimental 2°HPT is clearly induced by the feeding of a high phosphorus diet (Bover et al., 1994).

An extensive study by Llach and associates (1985) demonstrated that restriction of dietary phosphorus in patients with moderate renal insufficiency is associated with higher serum ionized calcium concentration, normalized PTH levels, improved intestinal absorption, a significant increase in plasma $1,25(OH)_2D_3$ and an improved calcemic response to PTH. The presence of normal calcitriol levels in this case of renal failure prior to dietary phosphate restriction indicates that phosphate retention may either interfere with the action of the sterol (i.e. resistance) and/or that the patient was in a state of relative vitamin D-deficiency caused, at least in part, by a direct suppression of the renal 1-hydroxylase enzyme (Tanaka et al., 1973). The authors proposed that altered vitamin D production was responsible for abnormal intestinal absorption and the impaired calcemic response to PTH thus leading to hypocalcemia and hyperparathyroidism. This study therefore supports an indirect mechanism by which phosphate retention can lead to hypocalcemia and subsequent 2°HPT.

The issue becomes more complicated however by studies demonstrating that low phosphorus diets fed to dogs (Lopez-Hilker et al., 1990) or patients (Aparicio et al., 1993) with advanced chronic renal failure causes a reduction in circulating PTH independent of changes in levels of calcium and calcitriol. This perhaps suggests the existence of other potential mechanisms of control by phosphorus in regulation of parathyroid gland activity in chronic renal failure. In fact, hypophosphatemic rats with normal circulating concentrations of calcium and $1,25(OH)_2D_3$ have reduced levels of PTH mRNA (Kilav et al., 1995). Furthermore, the same study showed that a high phosphate diet is associated with elevated PTH gene expression. Finally, phosphate has also been shown to directly stimulate secretion of PTH in cultured rat parathyroid glands (Slatopolsky et al., 1995). Although these effects are believed to be direct, further studies will be required to establish the exact

mechanism(s) by which phosphate acts.

ii. Role of Altered Vitamin D Metabolism

The finding of reduced circulating levels of calcitriol in patients with mild to severe renal failure provides evidence for a role of altered vitamin D metabolism in 2°HPT. Because $1,25(OH)_2D_3$ has suppressive effects on parathyroid cell secretion, synthesis and proliferation, altered metabolism, specifically including diminished production and end-organ resistance, could result in direct enhancement of parathyroid gland activity.

Numerous *in vivo* studies provide direct physiological evidence for a causal role of $1,25(OH)_2D_3$ *per se* in the pathogenesis of parathyroid disease in renal failure. For example, PTH gene expression is elevated in an experimental animal model of chronic renal failure in which rats undergo a 5/6 nephrectomy (Shvil et al, 1990). While these rats have normal levels of calcium and phosphate, there is a concomitant reduction in the levels of $1,25(OH)_2D_3$. Moreover, injection of calcitriol into the experimental group decreases the elevated PTH mRNA levels, whereas short-term increases or decreases in serum calcium have no effect suggesting that perhaps the hyperplastic glands remain responsive to $1,25(OH)_2D_3$ but not to calcium (Shvil et al., 1990). These results may also imply that normal levels of $1,25(OH)_2D_3$ are required for elicitation of a proper response to calcium by the parathyroid gland.

Feeding vitamin D-deficient diets to animals is a means of achieving low circulating levels of $1,25(OH)_2D_3$ and subsequent development of 2°HPT as reflected by increased PTH gene expression (Naveh-Many et al., 1990). Furthermore, Szabo et al. (1989) reported that parathyroid cell hyperplasia rather than hypertrophy is responsible for the increase in parathyroid gland weight in uremic rats. Administration of $1,25(OH)_2D_3$ to these rats resulted in a dose-dependent inhibition of parathyroid cell proliferation as was assessed by ^{3}H-thymidine uptake and analysis for mitotic activity. Hyperplasia could be prevented altogether if animals were treated daily with calcitriol immediately following induction of renal failure. Notably, treatment failed to completely reverse hyperplasia once it has already occurred.

The exact causes of abnormal vitamin D metabolism in 2°HPT are not known, however, a decrease in renal mass (Hsu et al., 1987), suppression of 1-hydroxylase activity by phosphate retention (Portale et al., 1989) and metabolic acidosis (Baran et al., 1982) are

believed to ultimately lead to diminished circulating levels of $1,25(OH)_2D_3$ and subsequent hypocalcemia due in part to reduced intestinal calcium absorption and a blunting of the calcemic effect of PTH on bone. Others (Bonjour et al., 1992), offer a more complicated explanation for the reduced levels of circulating calcitriol observed in early renal failure. In contrast to the aforementioned "trade-off hypothesis", this theory has a decrease in $1,25(OH)_2D_3$ instead of an increase in PTH secretion as the primary adaptive compensatory response to an alteration in intracellular phosphate pool. PTH hypersecretion would then be secondary to the decrease in the sterol and any concomitant effects it may have on other systems such as altered calcemic response to PTH.

Because of its known effects on parathyroid cell synthesis and proliferation, calcitriol has been used for many years to treat 2°HPT. In fact, administration of $1,25(OH)_2D_3$ to patients ameliorates their conditions by reducing the high levels of circulating PTH and improving bone disease (Andress et al., 1989; Slatopolosky et al., 1984). Unfortunately, the frequent hypercalcemic effects associated with high therapeutic doses of $1,25(OH)_2D_3$ and long length of treatment restricts the clinical usage of this compound. In this regard, the use of non-calcemic synthetic analogues such as 22-oxa-$1,25(OH)_2D_3$ are proving useful in chronic renal failure rats by inhibiting secretion (Kubrusly et al., 1993) and synthesis (Fukagawa et al. 1991) of PTH.

iii. Vitamin D Receptor Alterations

Many of the biological effects exerted by $1,25(OH)_2D_3$ are mediated by binding to its intracellular receptor, the VDR. Under normal circumstances, tissue specific changes to VDR gene expression, content and/or binding affinity are potential means of modulating the biological responsiveness to the sterol (Hunziker et al.,1982; Chen et al., 1986). Evidence exists to support the notion that such alterations may occur in chronic renal failure, thus contributing to end organ resistance and therefore a diminished responsiveness to the actions of $1,25(OH)_2D_3$. For instance, supraphysiological levels of calcitriol are required to lower PTH mRNA levels in a rat model of moderate renal failure (Fukagawa et al., 1991). Similarly, nephrectomized rats have a decreased intestinal calcium absorption response when injected with pharmacological doses of calcitriol (Walling et al., 1976). While such abnormalities have been attributed primarily to reduced calcitrol concentrations, normal

levels of the sterol have been reported in moderate renal failure (Cheung et al., 1983; Llach et al., 1988; Fukagawa et al., 1991; Bover et al., 1994b) consistent with the involvement of end organ resistance in 2°HPT. In the parathyroid, loss of the inhibitory actions of $1,25(OH)_2D_3$ may be a key factor in the progression of hyperparathyroidism, while in the intestine, an altered response could lead to hypocalcemia.

More extensive studies indicate that patients with chronic renal failure have a reduced parathyroid VDR content as compared to those with transplanted kidneys or parathyroid adenomas (Korkor et al., 1987). No differences exist however in the binding affinity of the VDR in any of these three groups. In addition, immunohistochemistry reveals a greater decrease in VDR density in the more clinically severe (nodular) form of hyperparathyroidism as compared to diffuse hyperplasia (Fukuda et al., 1993). The heterogeneous distribution of the staining is characterized by a high concentration outside the nodules with little staining in the proliferating cells located inside the nodules. A negative correlation seems to exist between the amount of staining and the weight of the gland. In experimental uremia, chronically uremic dogs have four times less parathyroid VDR content as compared to normal (Brown et al., 1989) whereas acutely uremic rats have decreased levels of parathyroid VDR protein at six days after induction of nephrectomy (Merke et al., 1987).

Despite the above studies, there is some controversy surrounding this issue. Szabo and associates (1991) re-examined VDR levels in uremic rats and found that these were increased rather than decreased. They explain that this different result was due to the use of protease inhibitors in their buffers and modification of their extraction procedure thereby preventing any unspecific degradation which may occur in uremia. Another study (Shvil et al., 1990) showed no change at the level of parathyroid VDR gene expression in an experimental rat model of chronic renal failure, however, because parathyroid gland enlargement was attributed to hyperplasia, they speculated that an actual decrease in VDR mRNA per cell had occurred.

Many studies dealing with VDR regulation, most of which involve $1,25(OH)_2D_3$ and calcium, have provided insight as to possible mechanisms causing such changes in chronic renal failure. *In vivo*, $1,25(OH)_2D_3$ increases parathyroid VDR mRNA levels (Naveh-Many et al., 1990; Russell et al., 1993). The physiological relevance of this induction may be to amplify the biological effects of the sterol via a positive-feedback

mechanism. Pharmacological doses of $1,25(OH)_2D_3$ also up-regulates the expression and protein levels (Costa and Feldman, 1986) of its own receptor in the intestine (Strom et al., 1989) and kidney of vitamin D-deficient rats. Other groups, however, have observed no alterations in VDR gene expression in intestine or kidney under similar conditions (Huang et al, 1989; Wiese et al., 1992). The descrepancies among these studies may have been caused by differences in the dosages administered (Naveh-Many et al., 1990) or in serum calcium levels. The latter point derives from a study showing that $1,25(OH)_2D_3$ supplementation to hypocalcemic rats does not change renal VDR levels, perhaps indicating that normal serum concentrations of calcium are requ: ed for vitamin D to have an effect on VDR levels (Sandgren and DeLuca, 1990). Furthermore, hypocalcemic rats with elevated serum levels of $1,25(OH)_2D_3$ induced by a dietary calcium restriction, do not have changes in their parathyroid VDR mRNA levels (Naveh-Many et al., 1990), reinforcing the complicated nature of the relationship between calcium and $1,25(OH)_2D_3$ in gene regulation. Evidence for post-transcriptional regulation comes from a study by Patel and associates (1994) in which the concentration of VDR protein was reduced, but VDR mRNA increased in the intestine of renal failure rats having normal values of calcium and reduced levels of $1,25(OH)_2D_3$. In the same study, administration of calcitriol in normal and chronic renal failure rats resulted in a biphasic reduction of intestinal VDR mRNA but an increase in VDR content at 48 hours suggesting an inhibitory effect at the post-transcriptional level. Ligand-induced stabilization may be a mechanism whereby differential changes at the mRNA and protein levels could occur (Wiese et al., 1992).

Other groups have provided additional evidence for an important role for calcium in the regulation of VDR. Hypocalcemia decreases VDR mRNA levels in chicken parathyroids (Russell et al., 1993), as well as renal VDR concentration in rats (Sandgren and DeLuca, 1990) regardless of the vitamin D status. In a recent study by Brown and associates (1995), parathyroid VDR mRNA levels in vitamin D-deficient rats were decreased while those in the intestine and kidney were unchanged. This indicates that such alterations may occur in a tissue-selective rather than a generalized manner. Furthermore, the fact that calcitriol administration to these rats increased parathyroid and kidney VDR gene expression but decreased it in the intestine points to tissue-specific regulation. Because the degree of the responses depended on the initial serum calcium concentrations, calcium was implicated as an important factor mediating the up-regulation of VDR gene expression

by $1,25(OH)_2D_3$.

Besides reduced levels of $1,25(OH)_2D_3$ and hypocalcemia, several other factors are believed to be responsible for reduced VDR content in chronic renal failure including elevated serum PTH levels and uremic toxins. PTH down-regulates VDR protein levels in rat osteosarcoma cells (ROS cells 17/2.8) and inhibits the $1,25(OH)_2D_3$ induced up-regulation of intestinal and renal VDR *in vivo* (Reinhardt et al., 1990). While these studies suggest that PTH may play a role in altering intestinal and renal VDR levels, it is questionable as to whether it plays a direct role in modulating those in the parathyroid, since to date, no PTH receptors have been found in this tissue (Brown et al.,1995).

Finally, it has been demonstrated that uremic ultrafiltrate injected into normal rats produces the same changes to VDR expression and content as those observed in rats with chronic renal failure (Hsu et al., 1993). Furthermore, uremic toxins inhibit uptake of the VDR into intestinal nuclei (Patel et al., 1994b) and impair VDR-VDRE interactions (Patel et al., 1995). It is unknown if structural abnormalities in the protein are responsible for these changes.

iv. Disturbances in Calcium-Regulated PTH Secretion

Alterations in calcium-regulated PTH secretion are thought to play a role in the development and progression of secondary hyperparathyroidism. Although the maximal suppressibility is within the normal range, evidence suggests that the set point, defined as the concentration of calcium required to suppress secretion by 50%, is sometimes elevated in parathyroid cells obtained from patients with 2°HPT (Brown et al., 1978,1982). In other words, the glands are still responsive to changes in extracellular calcium concentrations, but are less sensitive since higher calcium concentrations are required to suppress PTH secretion. In an attempt to elucidate the factors reponsible for set-point abnormalities in hyperplastic tissue, calcitriol administration to uremic patients was found to increase parathyroid gland sensitivity to calcium (represented by a decrease in set-point) (Delmez et al., 1989). However, utilizing dynamic *in vivo* tests, several groups have failed to observe set-point differences between normal subjects and patients with 2°HPT (Ramirez et al., 1993; Messa et al., 1994; Goodman et al. 1995). Likewise, no disturbances in set-point were observed in an animal model of 2°HPT induced by dietary means (Hendy et al., 1989).

In these cases, the absence of alterations in calcium-regulated PTH secretion might be caused by an increase in the cumulative basal cellular secretion due to increased parathyroid size.

Alterations with the recently cloned calcium sensing receptor (Brown et al., 1993) may give a clue as to whether problems exist in calcium-dependent signal transduction. To date, analysis of parathyroid tumors, including secondary hyperplasias, have failed to detect any mutations in the gene (Hosokawa et al., 1995). In a recent *in vivo* study (Rogers et al., 1995) calcium sensing receptor mRNA levels in rats were not modulated by calcium or vitamin D, indicating that changes in expression of this gene do not regulate the responsiveness of parathyroid gland under normal conditions. However, preliminary experiments reveal that changes at the level of mRNA may contribute to the altered sensitivity observed in 2°HPT. A reduction in calcium sensing receptor mRNA has been observed in parathyroids obtained from patients with 2°HPT compared with normal parathyroid tissue (Gogusev et al., 1995). Likewise, Kifor and associates (1995) have recently reported a reduction in calcium receptor protein content in uremic glands. Finally, evidence for post-receptor defects comes from the finding of abnormal adenylate cyclase activity in hyperplastic parathyroid tissue (Bellorin-Font et al., 1981). Further studies will be required to determine if such defects are directly associated with the CaR.

v. PTH Resistance

Evanson and co-workers (1966) provided some of the earliest evidence that resistance to PTH was involved in 2°HPT when they reported a reduced calcemic response to parathyroid extract in uremic patients. This phenomenon is believed to be an important contributor to the hypocalcemia and development of 2°HPT observed in moderate and severe renal failure (Massry et al., 1973; Llach et al., 1975). Under such conditions, the ability of PTH to properly exert its homoeostatic functions on distal cells in the bone, kidney and intestine, is greatly compromised.

Several uremic factors are believed to be responsible for PTH resistance including phosphate retention (Somerville and Kaye, 1979; Rodriguez et al., 1991; Bover et al., 1994b) and abnormal vitamin D metabolism (Lewin et al., 1982). Dietary phosphate restriction improves the calcemic response to PTH (Llach and Massry, 1985). Although this effect may be indirectly caused by an increase in circulating levels of $1,25(OH)_2D_3$ in mild renal

failure, such is not the case in the advanced form of the disease (Rodriguez et al., 1991) where calcitriol levels are not influenced. Others have demonstrated that administration of the sterol partially corrects the impaired response to PTH in parathyroidectomized experimental animals with acute (Massry et al., 1976) and chronic renal failure (Somerville et al., 1978). This issue is controversial however, since calcitriol supplementation does not always lead to an improvement of skeletal resistance (Somerville and Kaye, 1978; Galceran et al., 1987).

Several studies support receptor desensitization, possibly due to increased circulating levels of PTH, as an underlying mechanism responsible for PTH resistance and provide insight into possible defects occurring at the receptor and post-receptor levels. *In vitro*, PTH has the ability to decrease PTH-specific binding as well as PTH-stimulated cAMP accumulation in osteosarcoma cell lines (Mitchell and Goltzman, 1990; Ureña et al., 1994a). Utilizing a dietary model of 2°HPT in rats, Mitchell and co-workers (1988) demonstrated that the renal membranes of such animals not only have reduced PTH-binding capacity, but also post-receptor defects involving G-proteins. Furthermore, parathyroidectomy has been shown to completely restore skeletal responsiveness in azotemic dogs (Galceran et al., 1987) and rats (Bover et al., 1994a; Rodriguez et al., 1991) and decrease renal PTH resistance, (measured by changes in cAMP levels), in patients with renal failure (Lewin et al., 1985). There also seems to exist a reduction in basal and PTH-stimulated adenylyl cyclase activity in crude renal membrane preparations of rats with chronic renal failure (Ureña et al., 1994b) however, parathyroidectomy does not lead to an improved response suggesting that in this case, elevated PTH levels are not the cause of reduced function (Ureña et al., 1995).

vi. Progression of Parathyroid Hyperplasia: Histological Considerations

Parathyroid hyperplasia is classified histologically as being either nodular or diffuse. In the early stages of chronic renal failure, parathyroid gland hyperplasia is considered diffuse, representing a generalized polyclonal enlargement of the glands in response to extrinsic stimulations such as vitamin D deficiency and hypocalcemia. As the disease progresses and becomes more severe, nodular hyperplasia sets in. In comparison to diffuse hyperplasia, nodule-forming areas represent a more actively dividing group of cells which are believed to contribute to most of the increase in parathyroid gland weight. Since

numerous nodules can form in the four glands of a patient, each having a different clonal origin, nodular hyperplasia has been classified as being multiclonal in contrast to monoclonal (Parfitt, 1994). More recently however, monoclonality has been detected in glands which have been histologically defined as diffuse (Arnold et al., 1995). Therefore, based on this type of histological classification, it is impossible to predict whether true generalized hyperplasia or clonality exists. Only if stimulation persists do the glands become autonomous and have the same monoclonal status as seen in parathyroid adenomas. This phenomenon is referred to as tertiary hyperparathyroidism.

In this context, Fakuda and associates (1993) discovered that nodule-forming areas stain much less for VDR as assessed by immunohistochemistry compared to areas showing diffuse hyperplasia (Fakuda et al., 1993). It is worth mentioning that this uneven staining pattern may partially explain why there are so many conflicting results regarding altered parathyroid VDR expression in chronic renal failure. Furthermore, since nodules are considered areas of active proliferation, these results are consistent with the notion that actively dividing cells lose their differentiated characteristics.

vii. Role of Growth Factors

While many studies have focused on parathyroid cell gene regulation and expression, few have dealt with the factors responsible for parathyroid cell growth. As a result, the exact causes for uncontrolled cell growth in hyperparathyroid states are unknown. While $1,25(OH)_2D_3$ deficiency and perhaps hypocalcemia play a role in stimulating cellular proliferation in secondary hyperparathyroidism, a potential role for growth factors may exist.

Several growth factors have been the subject of studies in chronic renal failure however the link to the parathyroid gland is rarely considered. Compensatory renal hypertrophy is a phenomenon that occurs after the loss of kidney mass. In nephrectomized rats, there is an induction of many genes, including insulin-like growth factor I (IGF-1) in the remnant kidney undergoing compensatory growth (Fagin and Melmed, 1987). Because serum levels of this growth factor are not altered in this model of renal failure, the authors concluded that it was most likely acting locally in a paracrine or autocrine fashion rather than distally on parathyroid cells.

On a similar note, MEN1 is an autosomal dominant disease characterized by

neoplasia in the parathyroid glands, pancreatic islets and the anterior pituitary. Early data pointed towards a humoral cause for parathyroid hyperplasia in this setting since plasma isolated from patients with MEN1 was shown to have a mitogenic effect on cultured bovine parathyroid cells (Brandi et al., 1986). Further investigation revealed that this plasma had significantly elevated bFGF-like immunoreactivity in comparison to normal and that the source was most probably pituitary tumor (Zimering et al., 1993). A limited number of plasma samples were isolated from patients with 2°HPT and tested for mitogenic activity on parathyroid cells, however no significant increase in growth was observed. Such experiment are worth examining in greater detail taking into consideration the severity of the renal dysfunction as well as the parathyroid hyperplasia.

A humoral agent responsible for stimulating parathyroid growth in secondary hyperparathyroidism has yet to be found. It is probably more likely that cellular proliferation is stimulated by an increase in production of local growth factors and cytokines which is secondary to changes occurring in serum calcium and $1,25(OH)_2D_3$ levels and/or parathyroid VDR expression or function. For instance, using immunohistochemistry methods, Gogusev and associates (1994) found that parathyroid cells obtained from patients with 2°HPT stain positively for transforming growth factor-alpha mRNA and protein whereas those of normal individuals do not. Furthermore, cells obtained from parathyroid carcinoma (a more severe but rarer neoplastic syndrome) have a greater number of IGF-1 receptors as compared to adenomas and hyperplastic tissue obtained from patients with chronic renal failure (Tanaka et al., 1994). These changes may render the cells more responsive to external stimuli. Future investigations focusing on changes at the level of growth factors and their receptors in uremic hyperplastic glands will be of great interest with respect to abnormal parathyroid cell growth. A useful means of proceeding could be by the differential display method which allows for the detection of mRNAs that are differentially expressed in an experimental state as compared to control (Rafaeloff et al., 1996).

E. CHROMOGRANIN A

Chromogranin (CgA) is the principal member of the granin family of proteins which are present in all endocrine and neuroendocrine cells. It is an acidic glycoprotein that was first identified in adrenal medullary chromaffin cell granules (Blaschko et al., 1967). In the parathyroid gland, CgA or parathyroid secretory protein as it was originally termed by Kemper and associates (1974), is the other major secretory product besides PTH.

Although the precise function of granins are not known for certain, co-localization with the resident hormone in secretory granules along with their co-secretion suggests an involvement in modulation of cell secretory activity. Furthermore, their widespread distribution is likely indicative of their importance in such a cellular process. Many of the studies to date have focused on trying to elucidate CgA's precise function in cell secretion. Based on such studies and those characterizing its biochemical properties, it is believed to function in an intracellular as well as extracellular capacity.

Granins have been the subject of much recent attention when it was reported that the familial breast and ovarian cancer gene BRCA1 encodes a protein which shares many characteristics common to the granin family of proteins (Jensen et al., 1996). For instance, it contains a consensus granin sequence, is heavily glycosylated, has an acidic isoelectric point, and is apparently localized in secretory vesicles. Future functional studies will be required to confirm that BRCA 1 is indeed a granin.

i. Extracellular Functions

Extracellularly, it is postulated that CgA serves as a precursor of biologically active peptides that inhibit endocrine cell secretion by binding an as of yet uncharacterized cell surface receptor(s) (Galindo et al., 1992). The existence of pairs of basic amino acids serving as possible cleavage sites in the CgA molecule support this notion, as does the requirement of extracellular processing of the CgA molecule for biological effectiveness (Fasciotto et al., 1992; Simon et al., 1988).

Tatemoto and co-workers (1986) discovered that a 49 amino acid peptide called pancreastatin, isolated from porcine pancreas, was able to inhibit glucose-stimulated insulin release from porcine pancreatic cells. Its primary structure was found to be identical to the

sequence of residues 240 to 288 of porcine CgA (Iacangelo et al., 1988), suggesting that pancreastatin is derived by proteolytic cleavage of the parent CgA molecule and may act either in an autocrine or paracrine fashion. With regards to the parathyroid gland, addition of pancreastatin to culture medium inhibits low calcium-stimulated PTH and CgA secretion from porcine parathyroid cells *in vitro* (Fasciotto et al., 1989) and decreases the rate of transcription and stability of their respective mRNAs thus indicating a complex mechanism of action for this fragment (Zhang et al., 1994). Interestingly, antibodies directed against CgA or pancreastatin potentiate the low-calcium PTH and CgA secretion (Fasciotto et al., 1990), previously thought to be at a maximal level. Despite these physiological effects, little pancreastatin immunoreactivity has been found in intracellular fractions obtained from bovine parathyroid cells (Drees and Hamilton, 1992) supporting the theory that little processing of CgA takes place in this tissue (Barbosa et al., 1991). One possible explanation is that the *in vivo* processing of CgA occurs by neighboring cells of a different type which could then act on the parathyroid cells. Tissue specific processing could be attributed to differences in expression of certain proteases and the variation and extent of tissue processing of CgA may be indicative of tissue-specific functions that this protein may have.

In addition to pancreastatin, other CgA-derived peptides shown to have biological activity include: (a) ß-granin, originally isolated from rat insulinoma cells (Hutton et al., 1987), shown to inhibit bovine parathryoid cell secretion of both PTH and CgA *in vitro* (Drees et al., 1991) and shares exact homology with the amino terminus of CgA (amino acids 1 - 114); (b) parastatin, identical to porcine $CgA_{(347-419)}$, generated by digestion of CgA by endoproteinase Lys-C, demonstrated to inhibit low-calcium stimulated PTH secretion from parathyroid cells (Fasciotto et al., 1993), however at a much higher concentration as compared to pancreastatin; (c) vasostatin [CgA (1-76)], a peptide that has inhibitory vasoactive actions on blood vessels (Aardal and Helle, 1992) as well as parathyroid cell secretion (Russell et al., 1994); and (d) chromostatin [$CgA_{(124-143)}$] which has been shown to inhibit chromaffin cell secretion of catecholamines (Galindo et al., 1991). It should be mentioned that controversy surrounds this last study stemming from the fact that many of the observed effects were due to a contaminant formed during the chemical synthesis of the chromostatin peptide.

In conclusion, the above studies implicate CgA as a precursor of biologically active peptides whose main purpose may be to modulate secretion of the principal hormone(s) in

endocrine cells via an autocrine and/or paracrine mechanism.

ii. Intracellular Functions

Indirect experimental evidence supports CgA playing an intracellular role in sorting, processing and packaging of hormones into secretory granules of the regulated pathway and helping in the formation of secretory vesicles.

Certain physicochemical properties, such as its ability to bind calcium with low affinity, but high capacity (Reiffen and Gratzl, 1986: Gorr et al., 1988; Leiser and Sherwood, 1989) might allow CgA to mediate certain secretory processes. Furthermore, since low pH negatively influences calcium binding and has been shown to affect the conformation of CgA (Yoo and Albanesi, 1991), environmental conditions may dictate how this protein functions. The finding that calcium enhances binding of CgA to catecholamines (Videen et al., 1992) and cellular membranes (Settleman et al., 1985; Leiser and Sherwood, 1989; Gorr et al., 1988), combined with the tendency of CgA molecules to aggregate under conditions of low pH and high calcium (Yoo et al., 1991) such as those found in the trans-golgi apparatus, implicates CgA as a possible contributor to granule condensation and the packaging of relevant hormones destined to be secreted. Further evidence pointing to a role for CgA in sorting and secretion of hormones via the regulated secretory pathway, involves its ability to selectively aggregate with a secreted polypeptide such as PTH rather than albumin, a constitutively secreted protein (Gorr et al., 1989).

Due to the presence of basic amino acids, it is conceivable that CgA could also compete as a substrate for endopeptidases thereby modulating processing of resident prohormones. In fact, using an *in vitro* assay system, addition of CgA inhibited IRCM-serine protease activity in the processing of pro-opiomelanocortin (POMC) and proenkephalin (Seidah et al., 1987).

iii. CHROMOGRANIN A GENE REGULATION

Studies dealing with the regulation of CgA biosynthesis and secretion have provided further insight into its numerous postulated functions, especially in parathyroid cells where

CgA, like PTH, is regulated by calcium and $1,25(OH)_2D_3$. Parathyroid cell secretion of both CgA and PTH has been shown to be increased under hypocalcemic conditions and decreased by high extracellular calcium concentrations *in vitro* (Kemper et al., 1974; Morrissey and Cohn, 1979). With regards to synthesis, several groups have reported no effect on CgA mRNA levels by changes in medium calcium concentration in cultured bovine parathyroid cells (Russell et al.,1990; Mouland and Hendy, 1991). On the other hand, different results were obtained by Zhang and co-workers (1993) who demonstrated that increased extracellular calcium concentrations causes a reduction in CgA mRNA levels in primary cultured porcine parathyroid cells. This descrepancy may be related to a species difference or in the manner by which the cells were prepared.

Much work has focused on the regulation of parathyroid CgA by $1,25(OH)_2D_3$. *In vitro* studies using bovine parathyroid cells indicate that while exogenously added $1,25(OH)_2D_3$ suppresses the release of PTH into the medium (Silver et al., 1985), it increases secretion of CgA (Ridgeway and MacGregor, 1988; Russell et al., 1990; Mouland and Hendy, 1991). It also enhances CgA mRNA levels in a time- and dose-dependent manner by acting directly on gene transcription (Russell et al., 1990; Mouland and Hendy, 1991) rather than affecting mRNA stability (Mouland and Hendy, 1991). More specific details about other various levels of regulation were discovered when studies were performed in an attempt to explain the larger relative increase in CgA mRNA expression as compared to CgA protein synthesis upon stimulation by $1,25(OH)_2D_3$ (Mouland and Hendy, 1992). It appears that the sterol reduces the rate of translation of CgA mRNA specifically by increasing the ribosome transit time of CgA mRNA.

Other steroid hormones which regulate CgA gene expression in a physiological manner include estrogen and glucocorticoids. *In vitro* experiments (Anouar and Duval, 1992) demonstrated that estrogen decreases rat pituitary CgA mRNA and protein levels while increasing those of LH and FSH. Moreover, pituitary CgA levels are increased after rats are subjected to ovariectomy (Lloyd et al., 1992; Anouar et al., 1991), but these are restored to basal levels after estrogen administration.

Glucocorticoids have also been shown to play an important role in regulating CgA *in vivo* (Fischer-Colbrie et al., 1988) and *in vitro* (Rausch et al., 1988). Early indication of such a regulation came from the finding that hypophysectomy in the rat causes a decrease in adrenal medulla CgA mRNA levels which was reversible with treatment of dexamethasone,

a synthetic glucocorticoid (Fischer-Colbrie et al., 1988). This confirms that the reduction of CgA levels was due to the lack of adrenocorticotropin (ACTH) and subsequent loss of production of glucorticoids by the adrenal medulla. Dexamethasone administration to normal rats increases anterior pituitary CgA levels as demonstrated by immunohistochemistry, immunoblotting and Northern blot analysis in a dose-dependent manner (Fischer-Colbrie et al., 1989). Interestingly, CgB levels in the same tissue were unchanged indicating differential regulation of the two granins.

In agreement with the above studies, dexamethasone up-regulates CgA expression in a rat pheochromocytoma cell line (PC12) (Rausch et al., 1988), via a direct effect on transcription initiation (Rozansky et al., 1994). The recent identification of a glucocorticoid response element in the promoter region of the rat CgA gene confirms the above studies (Rozansky et al., 1994). Finally, in the corticotrophic cell line, AtT-20, glucocorticoids increase CgA mRNA levels but decrease expression of POMC peptides (Wand et al., 1991).

The above studies clearly demonstrate opposite regulation of CgA and the native hormone in several cell types including the parathyroid cell and pituitary gonadotrophs and corticotrophs. It is postulated that the physiological relevance of such differential regulation by a single agent is to provide the cell with an additional mechanism of control for cell secretion of the principal hormone. In the parathyroid for instance, if we assume an extracellular action of CgA-derived peptides, an increase in CgA synthesis and secretion by $1,25(OH)_2D_3$ would allow for a modulatory role of CgA-derived peptides specifically by further inhibiting PTH secretion in addition to the direct effect of $1,25(OH)_2D_3$ in inhibiting PTH synthesis.

An example of the complexity of CgA gene regulation by steroid hormones is observed with dexamethasone. Whereas this steroid increases PTH mRNA levels under low and high medium calcium concentrations *in vitro* in cultured bovine parathyroid cells, it modulates CgA gene expression in both directions depending on the medium calcium concentration (Zhang et al., 1993). In the presence of an elevated calcium concentrations, dexamethasone increases CgA mRNA levels and secretion while under low calcium conditions, synthesis and secretion are decreased by the steroid.

iv. CHROMOGRANIN A IN DISEASE STATES

An interesting area of research has focused on the role of CgA in disease states. O'Connor and Bernstein (1984) were the first to develop a radioimmunoassay for human chromogranin A purified from pheochromocytoma vesicles. They determined that serum CgA levels in patients with pheochromocytoma were much higher than normal, thus concluding that such measurements might be useful to assess abnormal sympathoadrenal activity, such as seen in patients suffering from pheochromocytoma. These results prompted O'Connor and Deftos (1986) to measure serum CgA levels in patients suffering from a wide variety of neoplastic disorders. They determined that many patients with endocrine and neuroendocrine tumours, including parathyroid adenoma, primary parathyroid hyperplasia, medullary thyroid carcinoma and pancreatic islet-cell tumor, had increased levels of immunoreactive serum CgA suggesting that this protein is a useful marker in diagnosing endocrine neoplasms. Interestingly, subjects with secondary hyperparathyroidism had much more elevated levels of plasma CgA (64-fold normal) as compared to those with parathyroid hyperplasia (28-fold) and adenoma (4-fold). A later investigation by Hsiao et al., (1990) concluded that the dramatic rise in CgA levels observed in secondary hyperparathyroidism was proportional to the severity of renal dysfunction and independent of parathyroid status pointing to an important role of the kidney in the clearance of this protein. Furthermore, it appears that the predominant circulating forms in uremia consist of fragments of the parent CgA molecule. The functional consequences of this are unknown.

Further evidence indicating that the parathyroid is not the source of elevated circulating CgA levels *per se*, comes from a study examining protein levels in parathyroid adenomas as well as sporadic and familial cases of parathyroid neoplasias (Nanes et al., 1989). Normal levels of plasma CgA were measured in most cases of primary hyperplasia, suggesting that elevated plasma CgA concentrations are not a general feature of primary hyperparathyroidism. Furthermore, parathyroidectomy did not cause a decrease in circulating levels of CgA in adenomas and hyperplasias but did in Familial multiple endocrine neoplasia type 1 (FMEN-I) patients who had Zollinger-Ellison syndrome. Changes in CgA levels correlated with gastrin concentrations rather than with indices of parathyroid function due to the indirect stimulatory effect of PTH on gastrin synthesis. At

the level of gene expression, Levine et al. (1990) found no change in CgA mRNA levels in parathyroids from patients with primary neoplasia (adenomas and FMEN1) supporting the findings of Nanes and associates (1989).

Since circulating $1,25(OH)_2D_3$ levels are usually elevated in primary hyperparathyroidism, it would follow that CgA mRNA and secretion also be increased under these conditions. One possible explantation for this inconsistency may be a failure of the hyperplastic gland to respond normally to calcitriol due to vitamin D resistance. Another possible factor is that the contribution of the parathyroid gland to the overall circulating CgA pool is relatively small.

V. THE CHROMOGRANIN A GENE

The human chromogranin A gene is a single copy gene that spans 15 kilobases. The organization as well as the relative sizes of the eight exons is similar among human, bovine, rat and mouse. Promoter activity can be tested by fusing a gene sequence upstream of a promotorless expression reporter gene, usually luciferase, growth hormone or chloramphenicol acetyl transferase (CAT). While it is known that activators of protein kinase A (e.g. forskolin) and protein kinase C (e.g. phorbol esters) stimulate CgA synthesis (Murray et al., 1988a, 1988b), promoter studies can yield more specific information on gene regulation. With regards to CgA, an important question has centered on what confers its neuroendocrine cell-specific expression. Preliminary evidence demonstrated that a construct composed of 2300 base pairs of the human 5' flanking region has strong promoter activity in several neuroendocrine cells (AtT20, TT, PC12, GH4C1), but is inactive in nonendocrine cells (e.g. COS-7, NIH-3T3) (Mouland et al., 1994). Sequential 5' end deletion of this fragment has revealed an 87-bp fragment spanning -55 to +32 relative to the transcription start site which is able to confer neuroendocrine cell-specific expression (Mouland et al., 1994). Further studies have demonstrated that an 18 bp fragment, centered around the CRE (cyclic AMP-responsive element) and spanning the region -56 to -39 can confer specificity. This minimal promoter was found to be as active as the full length construct (-2300 to +32). Mutation of the CRE which is contained within this fragment reduced transcriptional acitivity in neuroendocrine cells but not non-neuroendcrine cells (Bevan et al., 1995).

Wu and associates (1994) have been working on the mouse chromogranin A gene

promoter and using promoter deletion constructs defined a minimal neuroendocrine-specific element between -77 and -61 bp which is as active as the full length (-4.8 kb) construct. The finding of a conserved CRE within the region spanning -77 to -61 led Wu and co-workers (1995) to introduce deletions or point mutations within the CRE. These resulted in the complete loss of activity in neuroendocrine cells, similar to what Bevan and associates had observed in the human gene constructs.

The fact that the CRE site and TATA box are conserved in two other members of the granin family; CgB and secretogranin II probably reflects the importance of these regions as regulatory elements and perhaps as playing a role in cell specific expression of granins. Conservation between the human and bovine proximal promoter seqences, and to a lesser extent the mouse gene, probably reinforces this aspect (Mouland et al., 1994). Because non-endocrine specific genes may contain functional CRE's, the presence of a CRE is probably not the sole factor responsible for endocrine-specific expression of CgA and the other granins. Although the CRE alone increases basal promoter activity in neuroendocrine cells, the activity is far less than that of the full length 4.8 kb construct, indicating that additional regulatory elements found in the distal promoter are essential for the proper expression of CgA. Electrophoretic mobility shift assays using oligonucleotides spanning the CgA CRE region revealed the formation of protein-DNA complexes when mixed with nuclear extracts of both neuroendocrine (AtT20) and non-neuroendocrine cells (NIH3T3) (Wu et al., 1994; 1995). Antibodies directed towards CRE-binding protein (CREB) confirmed that CREB was part of the complex in both cell types indicating that CREB *per se* is not the protein that is responsible for cell-specific expression. A possible clue might come from the anti-CREB supershift data which reveal two complexes in the NIH3T3 extracts that are not present in the AtT20 extracts. The authors speculate that this may be caused by factors or repressors present in non-endocrine cells which bind CREB and inhibit its stimulatory action. Further work will be required to elucidate the factors responsible for conferring cell-specific regulation.

Nolan and associates (1995) reported that an upstream sequence between -726 and -455 (referred to as the distal regulatory region) in the 5' human CgA gene flanking region increases basal transcription considerably but only in neuroendocrine cells. They were able to attribute the transcriptional enhancement to a DNA element spanning the region -576 to -550 which they refer to as the distal regulatory element (DRE) and discovered an AP-1-like

element within this stretch of DNA. Antibodies directed against c-Jun and c-Fos resulted in a supershift of bands in electromobility shift assays using nonendocrine cell extracts. In contrast, it appears that AP-1 is not the major binding complex in neuroendocrine cells. The important proteins that bind this element in neuroendocrine cells have yet to be identified. The studies of Nolan and co-workers suggest a role for a distal regulatory region which may work in concert with the CRE in the BEN neuroendocrine cell line. Whether this type of mechanism operates in other neuroendocrine cells remains to be demonstrated.

PART 2:

IN VIVO REGULATION OF PARATHYROID CHROMOGRANIN-A GENE EXPRESSION

INTRODUCTION

Chromogranin-A (CgA), and parathyroid hormone (PTH) are the two major secretory products of the parathyroid gland (Kemper et al., 1974; Cohn et al., 1982). CgA, the principal member of the granin family of acidic glycoproteins, is widely distributed in endocrine and nervous tissue (Winkler et al., 1992). Because granins are found costored and are cosecreted with the resident hormone or neurotransmitter, they are believed to be important for cell secretory activity although their precise function(s) remain to be fully elucidated (Winkler et al., 1992; Hendy et al., 1995).

Extracellular calcium and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are the main regulators of parathyroid gland synthetic and secretory activity (Russell et al., 1983; Brookman et al., 1986; Cantley et al., 1985; Nygren et al., 1988; Silver et al., 1985, 1986; Russell et al., 1986). *In vitro* studies using bovine parathyroid cells in primary culture have shown that the transcription and secretion rates of PTH and CgA are regulated in opposite directions by $1,25(OH)_2D_3$ (Ridgeway et al., 1988; Russell et al., 1990; Mouland et al., 1991). The sterol decreases PTH (Silver et al., 1985, 1986; Russell et al., 1986), but increases CgA gene expression in a time- and dose-dependent fashion (Russell et al., 1990; Mouland et al., 1991). Regulation of PTH expression and secretion by calcium and $1,25(OH)_2D_3$ has also been studied using *in vivo* models (Silver et al., 1986, Naveh-Many et al., 1990; Yamamoto et al., 1989). However, no study to date has investigated the *in vivo* regulation of parathyroid CgA gene expression.

In hyperparathyroidism, secondary to chronic renal failure, abnormal changes in serum chemistries caused by renal insufficiency play a role in altering the normal regulation of synthesis and secretion of PTH (Llach et al., 1985; Lopez-Hilker et al., 1886; Bover et al., 194). Increased PTH gene expression and PTH secretion are believed to be attributable, at least in part, to reduced circulating levels of $1,25(OH)_2D_3$ (Shvil et al., 1990; Reichel et al., 1993). Although several studies have focused on the abnormal synthesis and secretion of PTH in renal failure, few have dealt with the expression of parathyroid CgA in such conditions.

Therefore, in order to gain a better understanding of the regulation of CgA gene expression *in vivo*, $1,25(OH)_2D_3$ was administered to normal rats and changes in parathyroid CgA mRNA expression were measured. Parathyroid CgA mRNA levels were

also evaluated in the uremic rat model to explore the potential role of altered expression of CgA in the pathophysiology of the secondary hyperparathyroidism.

MATERIALS AND METHODS

Animals.

Normal male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada), weighing 180 - 200 g, were fed a standard rodent chow (Ralston-Purina, LaSalle, Quebec, Canada) containing 1.01% calcium, 0.74% phosphorus and 3.3 IU vitamin D_3/g. All animal experiments were carried out according to institutional guidelines.

For the studies which investigated the CgA mRNA response to $1,25(OH)_2D_3$, rats fed the above diet were divided into five groups. Group 1 was injected intraperitoneally (i.p.) with vehicle (propylene glycol 0.2 ml/100 g body weight) at 48 and 24 hours prior to sacrifice. Groups 2 - 4 received $1,25(OH)_2D_3$ at doses of 10, 50 or 250 pmol/100 g body weight. Group 5 received 25 $(OH)D_3$ at a dose of 250 pmol/100 g body weight.

Uremia was induced in the rats in the chronic renal failure studies by a one-stage, 5/6 nephrectomy procedure under pentobarbital anesthesia (60 mg/kg, i.p.). Control animals underwent a sham operation which involved exposure of the kidneys and subsequent closure of the two separate flank incisions. The sham-operated and 5/6 nephrectomized rats were either maintained on the standard chow or were fed a high phosphorus diet which contained 0.6% calcium, 1.4% phosphorus and 1.0 I.U. vitamin D_3/g (TD94238, Teklad, Madison, WI, USA). Five weeks after surgery, the rats were anesthetized with pentobarbital, a blood sample was collected by cardiac puncture and the parathyroid and thyroid glands were microdissected separately.

Plasma Analyses

Serum total calcium, phosphate, creatinine and urea nitrogen levels were measured using Autoanalyzer techniques. Serum PTH levels were assessed using a sensitive two-site immunoradiometric assay kit for rat PTH-(1-34) which detects the intact PTH molecule and amino-terminal fragments equally (Immutopics, San Clemente, CA. USA; Schultz et al., 1994). Serum PTH levels are reported as pg-equivalents of rat PTH-(1-34)/ml. Serum $1,25(OH)_2D_3$ levels were measured using a commercial radioreceptor assay kit (Nichols, San Juan Capistrano, CA, USA; Reinhardt et al., 1984).

RNA Extraction

Parathyroid total RNA from two parathyroid glands was obtained by the following method: Two microdissected glands from an individual animal were homogenized using a 22 1/2 gauge needle and 3 ml syringe in 300 µl 5 M guanidine isothiocyanate, 10 mM EDTA, 50 mM Tris, pH 7.5, containing 0.8% (v/v) 2-mercaptoethanol. Five volumes of 4 M LiCl was added and the mixture was left overnight at 4°C. The solution was centrifuged at 10 000 x g, 30 min, 4°C and the pellet was resuspended in 300 µl 50 mM Tris-HCL, pH 7.5, 5 mM EDTA, 0.5 % SDS, 150 µg/µl proteinase K. Digestion was carried out at 37°C for 1 hour. Further purification was performed by extraction with phenol-chloroform-isoamyl alcohol (50:50:1). To the recovered aqueous phase, 1/10 volume of 3 M sodium acetate, pH 5.2 was added. The RNA was precipitated overnight with ethanol at -20°C. The pellet, obtained by centrifugation, was lyophilized and resuspended in 10 µl DEPC-treated H_2O. Thyroid total RNA was obtained using the same method. Total RNA was isolated from rat pheochromocytoma PC-12 cells as previously described (Mouland and Hendy, 1991) and used as a control for chromogranin B mRNA expression.

Northern Blot Analysis

The entire volume (10 µl) of RNA was fractionated on a 1% agarose-0.66 M formaldehyde gel, transferred to Nytran by blotting, and fixed by baking the filter at 80°C for two hours under vacuum.

Prehybridization and hybridization of Northern blots were performed in 1% BSA, 7% SDS, 0.5 M $NaPO_4$ (pH 6.8), 1 mM EDTA. The probes were as follows: 1) CgA; a synthetic (36-mer) oligonucleotide (5'-AGTGTCCCCTTTTGTCATAGGGCTGTTC ACAGGAAG-3') complementary to the rat CgA mRNA sequence encoding amino acids +1 - +12 (Parmer et al., 1989): 2) Chromogranin B (CgB); a synthetic (39-mer) oligonucleotide (5'-AGTCACCATTTCTTCATTGTGGTCCCTGTTATCCACTGG-3') complementary to CgB mRNA sequence encoding amino acids +2 - +14 (Fischer-Colbrie et al., 1988): 3) Thyroglobulin (Tg); a synthetic (30-mer) oligonucleotide (5'-CAACAAAGTCGAGACCCACAAGAC CAAGGT-3') complementary to the rat Tg mRNA sequence encoding amino acids +3 - +12 (Musti et al., 1986): 4) 28S rRNA; a synthetic (30-mer) oligonucleotide (5'- CGTCGCTATGAACGCTTGGCCGCCACAA GC-3') complementary to nucleotides 4131-4160 at the 3' end of rat 28S ribosomal RNA

(Hadjiolov et al., 1984). 5) PTH; a 400-bp PstI-XbaI restriction fragment containing most of intron B and exon II of the rat PTH gene (Heinrich et al., 1984) was subcloned into pGEM3 and kindly provided by Dr. Gerhard Heinrich. Oligonucleotides were 5'-end-labelled using [^{32}P]adenosine-5-triphosphate and T4 polynucleotide kinase, and the cDNA insert was labelled with [^{32}P]deoxy-CTP by the random primer method to a specific activity of 10^9 cpm/μg (Feinberg et al., 1983).

Membranes were washed four times at 65°C in 0.5% BSA, 5% SDS, 1 mM EDTA, 40 mM $NaPO_4$ (pH 6.8), and then four times at 65°C in 1% SDS, 1 mM EDTA, 40 mM $NaPO_4$ (pH 6.8). They were subsequently exposed to Kodak XAR film (Eastman Kodak, Rochester, NY, USA). Relative intensities were assessed using a LKB Ultroscan XL densitometer (LKB, Baie d'Urfe, Quebec, Canada). The signals for CgA and PTH mRNA were expressed relative to that for 28S rRNA.

Statistical Analysis

Data are reported as mean ± SEM. The results from the $1,25(OH)_2D_3$ response studies were initially subjected to analysis of variance (ANOVA). The significance of differences from control, vehicle-injected rats was then determined using Dunnett's multiple comparison test. The significance of differences in the chronic renal failure studies was determined using Student's *t*-test. A P value of <0.05 was taken to indicate a significant difference in both studies.

RESULTS

Successful Microdissection of Parathyroid Gland Free of Thyroid Tissue. As shown in Fig. 1, parathyroid gland RNA was virtually negative for Tg mRNA. Thus, the parathyroid glands were obtained essentially free of contaminating thyroid tissue. This was confirmed by histological analysis. Glands were randomly isolated, sectioned (1 μm thickness) and stained with methylene blue. Examination under light microscopy showed parathyroid glands surrounded by some small amounts of loose connective tissue and very little, if any, thyroid tissue (data not shown).

Chromogranin A, but not Chromogranin B, is Expressed in the Parathyroid Gland. Several neuroendocrine tissues express both CgA and CgB. Therefore, we tested for both CgA and CgB mRNAs in the rat parathyroid gland. Whereas the parathyroid expressed a high level of CgA mRNA it was negative for the related granin family member, CgB (Fig. 2). A high level of CgB mRNA was demonstrated in rat pheochromocytoma cells (Fig.2).

1,25(OH)$_2$D$_3$ Increases Parathyroid CgA mRNA Levels in a Dose-Dependent Manner. Serum calcium levels were increased significantly by the 50 and 250, but not the 10 pmol/100 g doses of 1,25(OH)$_2$D$_3$, whereas phosphate levels were increased significantly only by the highest dose (Table 1). In contrast, PTH levels were decreased significantly with all doses of 1,25(OH)$_2$D$_3$. Serum 1,25(OH)$_2$D$_3$ remained significantly elevated in the rats that received the 50 and 250 pmol doses at 24 hours after the second injection.

As shown in Fig. 3, 1,25(OH)$_2$D$_3$ increased CgA gene expression in a dose-dependent manner. Parathyroid CgA mRNA levels in rats injected with 50 and 250 pmol/100 g doses were 2.5-fold and 5-fold higher, respectively, compared to those in rats injected with vehicle alone. Likewise, 1,25(OH)$_2$D$_3$ regulated PTH gene expression in a dose-dependent manner, but in the opposite direction. Reductions in preproPTH mRNA levels of 65% and 95%, respectively, were observed in rats injected with 50 and 250 pmol doses (Fig. 3). In contrast, neither PTH nor CgA mRNA levels were altered in the parathyroid glands of rats injected with 25-hydroxyvitamin D$_3$ [25(OH)D$_3$] at doses up to 250 pmol/100 g (data not shown).

Parathyroid CgA mRNA is less sensitive to 1,25(OH)$_2$D$_3$ than PTH mRNA. Rats injected with 10 pmol/100 g 1,25(OH)$_2$D$_3$ did not modulate their parathyroid CgA mRNA levels, whereas PTH mRNA levels responded with a 37% decrease relative to control (Fig. 4).

Parathyroid CgA mRNA Levels are Decreased in Normocalcemic Uremic Rats with Reduced 1,25(OH)$_2$D$_3$ levels. Five weeks after 5/6 nephrectomy, rats fed the normal diet had elevated serum creatinine, urea nitrogen and PTH levels compared to sham operated controls (Table 2). However, serum calcium and phosphate concentrations were normal despite reduced serum 1,25(OH)$_2$D$_3$ levels. Parathyroid CgA mRNA levels in the uremic rats were approximately 50% those in the sham operated rats, whereas preproPTH mRNA levels were elevated 4-fold (Fig. 5).

Parathyroid CgA mRNA Levels are Increased in Hypocalcemic Uremic Rats with Reduced 1,25(OH)$_2$D$_3$ levels. Uremic rats fed the high phosphate diet had elevated serum levels of creatinine, urea nitrogen and PTH (Table 3). Serum calcium and 1,25(OH)$_2$D$_3$ levels in the uremic rats were lower compared to control, whereas phosphate levels were significantly higher. 5/6 Nephrectomized rats demonstrated 2.5-fold increases in both parathyroid CgA and PTH mRNA levels (Fig. 6).

Table 1. Serum Biochemistries of Vehicle- and 1,25(OH)$_2$D$_3$-Injected Rats.

		+ 1,25(OH)$_2$D$_3$ (pmol/100 g)		
	Control	10	50	250
Calcium (mmol/l)	2.57 ± 0.02	2.52 ± 0.02	3.02 ± 0.08**	3.18 ± 0.06**
Phosphate (mmol/l)	2.91 ± 0.15	3.41 ± 0.25	3.63 ± 0.16	3.90 ± 0.35**
PTH (pg/ml)	42 ± 6	17 ± 4*	8 ± 2**	6 ± 1**
1,25(OH)$_2$D$_3$ (pg/ml)	106 ± 4	97 ± 4	144 ± 7**	182 ± 12**

Data are mean ± SEM (n=8-12/group).
Significantly different from control; *$P < 0.05$; **$P < 0.01$.

Table 2. Serum Biochemistries of Sham Operated and 5/6 Nephrectomized Rats Fed a Normal Diet for 5 weeks after Surgery.

	Sham Operated	5/6 Nephrectomized
Creatinine (µmol/l)	24.8 ± 1.9	53.9 ± 6.5**
Urea Nitrogen (mmol/l)	5.0 ± 0.7	10.8 ± 2.9*
Calcium (mmol/l)	2.53 ± 0.03	2.56 ± 0.07
Phosphate (mmol/l)	2.7 ± 0.2	2.5 ± 0.2
PTH (pg/ml)	37 ± 4	71 ± 5*
$1,25(OH)_2D_3$ (pg/ml)	118 ± 6	63 ± 8**

Mean ± SEM (n=5-8/group).
Significantly different from sham-operated controls; Student's *t* test, $*P < 0.05$;$**P < 0.01$.

Table 3. Serum Biochemistries of Sham Operated and 5/6 Nephrectomized Rats fed a High Phosphorus Diet for 5 Weeks after Surgery.

	Sham Operated	5/6 Nephrectomized
Creatinine (μmol/l)	27.3 ± 1.7	48.5 ± 2.6**
Urea Nitrogen (mmol/l)	6.5 ± 0.5	12.1 ± 0.8**
Calcium (mmol/l)	2.49 ± 0.03	2.31 ± 0.03**
Phosphate (mmol/l)	2.7 ± 0.1	3.6 ± 0.2**
PTH (pg/ml)	121 ± 22	321 ± 42**
$1,25(OH)_2D_3$ (pg/ml)	107 ± 12	73 ± 4*

Mean ± SEM (n=5-8/group).
Significantly different from sham-operated controls; Student's *t* test, $*P < 0.05$;$**P < 0.01$.

Table 4. Summary of direction of changes in serum biochemistries and parathyroid CgA and PTH mRNA in uremic rats fed normal and high phosphate diets.

	Diet	
	Normal	High Phosphate
Serum		
Ca^{2+}	N*	↓
PTH	↑	↑
$1,25(OH)_2D_3$	↓	↓
Parathyroid		
PTH mRNA	↑	↑
CgA mRNA	↓	↑

N*, Normal; ↑, Increased; ↓, Decreased.

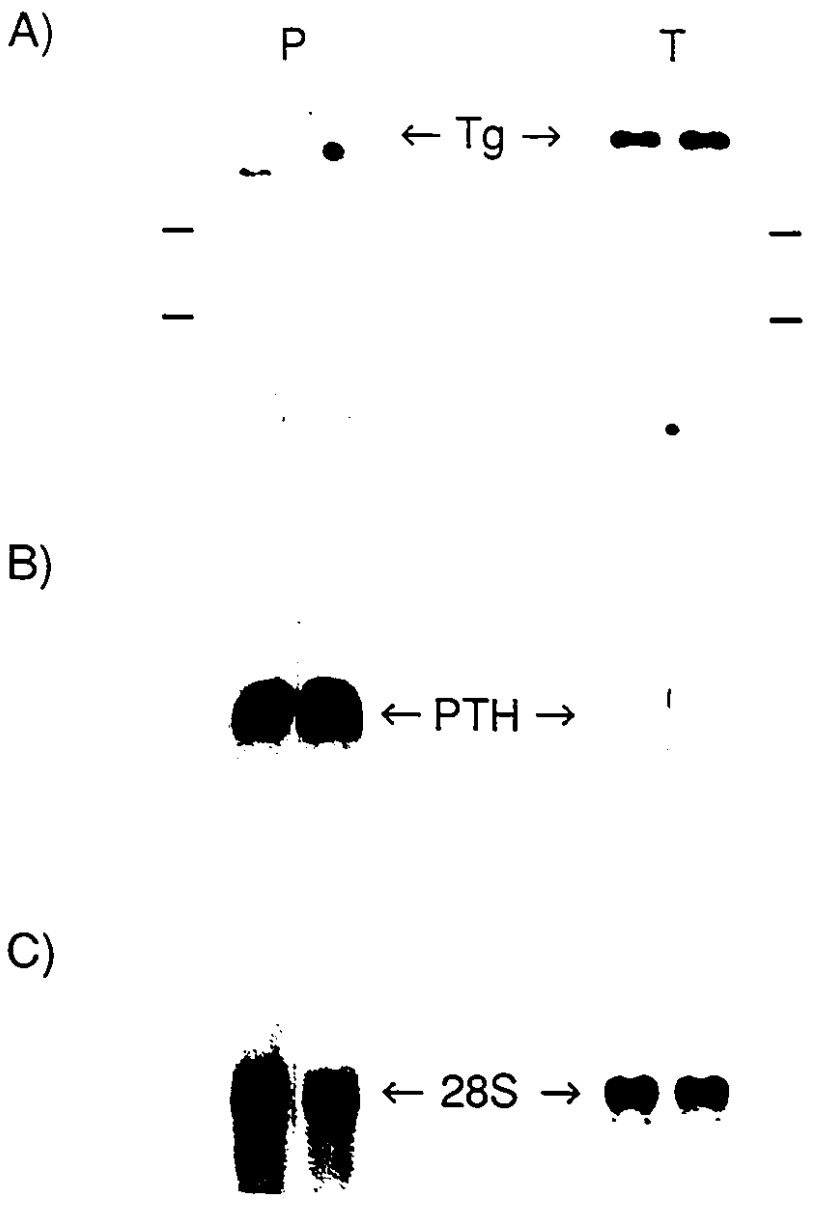

Figure 1. **Successful microdissection of parathyroid gland free of thyroid tissue.** Thyroglobulin (Tg) mRNA (Panel A), parathyroid hormone (PTH) mRNA (Panel B) and 28S ribosomal RNA (Panel C) were measured in parathyroid gland (P) and thyroid (T) by Northern blot analysis as described in Methods. In Panel A horizontal lines represent positions of 28S and 18S ribosomal RNA. Very low levels of Tg mRNA are present in Lane P, Panel A indicating virtually no parathyroid contamination on the microdissected parathyroid glands.

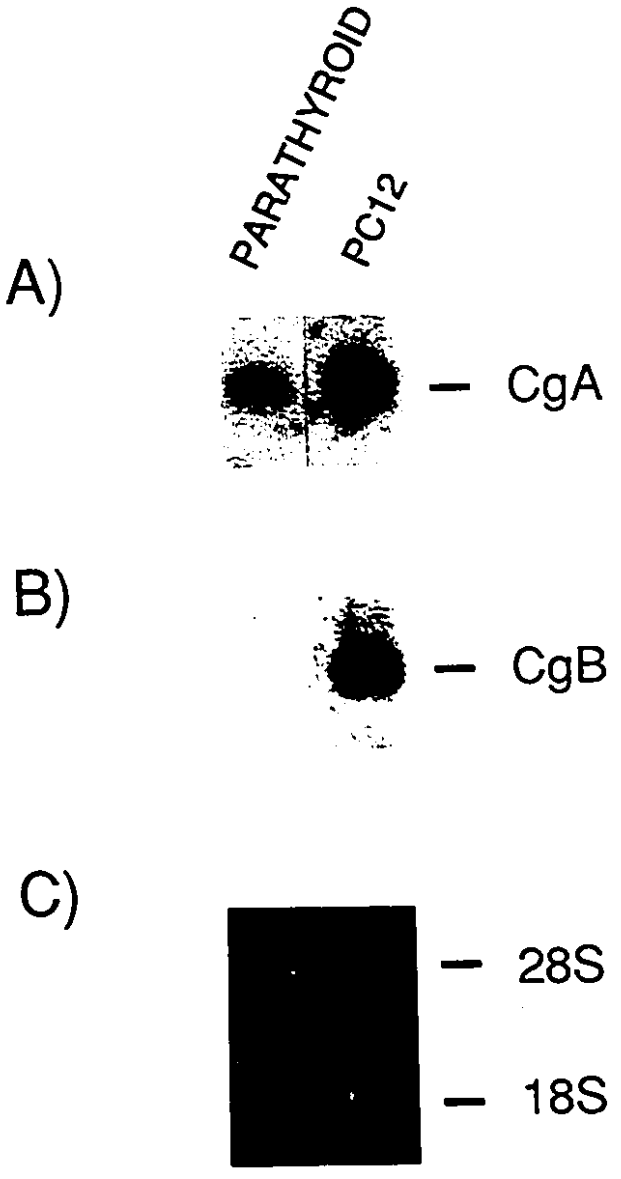

Figure 2. **CgA, but not CgB is expressed in the parathyroid.** (A) Rat parathyroid and rat pheochromocytoma PC12 demonstrated highly abundent expression of CgA, however (B), CgB was not expressed in parathyroid, only in PC12 cells. (C) Relative RNA quantity and quality was monitored by ethidium bromide staining of 28S and 18S ribosomal RNA.

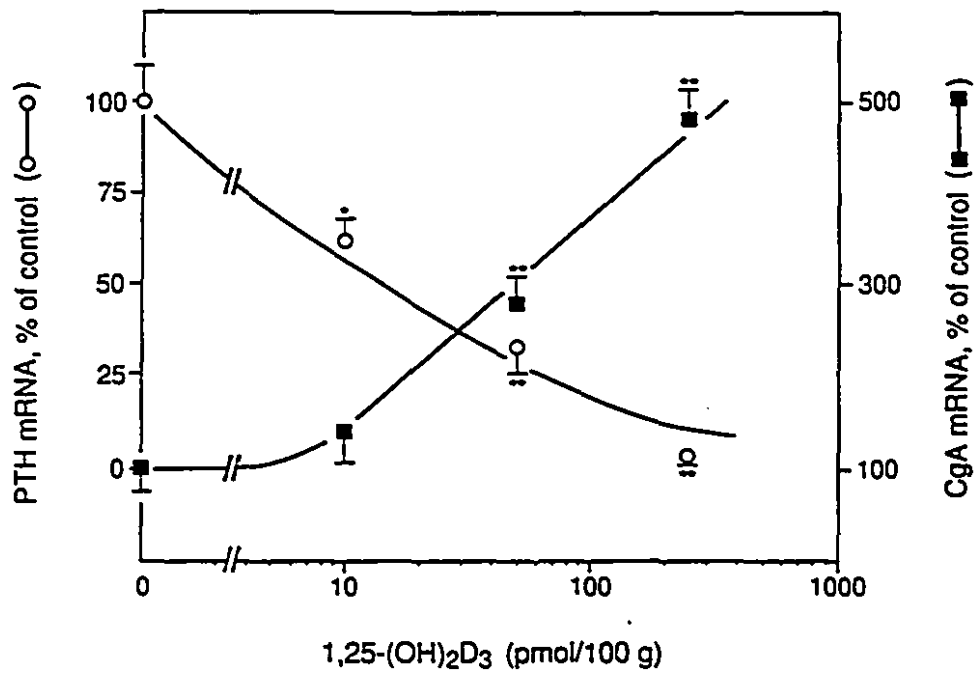

Figure 3. **1,25(OH)$_2$D$_3$ increases parathyroid CgA mRNA levels in a dose-dependent manner.** Northern blot analysis was conducted on parathyroid RNA from rats injected with either vehicle or 10, 50 or 100 pmol 1,25(OH)$_2$D$_3$/100g 48 h and 24 h before sacrifice. CgA, PTH mRNAs and 28S ribosomal RNA were measured as described in Methods. Relative CgA and PTH mRNA levels were assessed by densitometry and related to 28S ribosomal RNA. Each point is the mean ± SEM (n=5-8). Asterisks indicate a significant difference from control (C) values (*, $p < 0.05$, ** $p < 0.01$). Whereas PTH mRNA levels are suppressed, those of parathyroid CgA mRNA are increased by 1,25(OH)$_2$D$_3$.

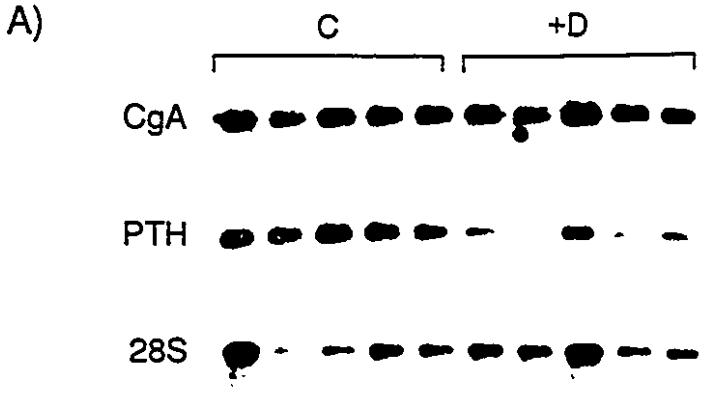

Figure 4. **Parathyroid CgA mRNA is less sensitive to $1,25(OH)_2D_3$ than PTH mRNA.** Autoradiographs of Northern blots of parathyroid RNA from rats injected with either vehicle alone (C) or 10 pmol $1,25(OH)_2D_3$/100 g (+D) before sacrifice. Whereas PTH mRNA is modulated by the 10 pmol/100 g dose, CgA mRNA is unresponsive.

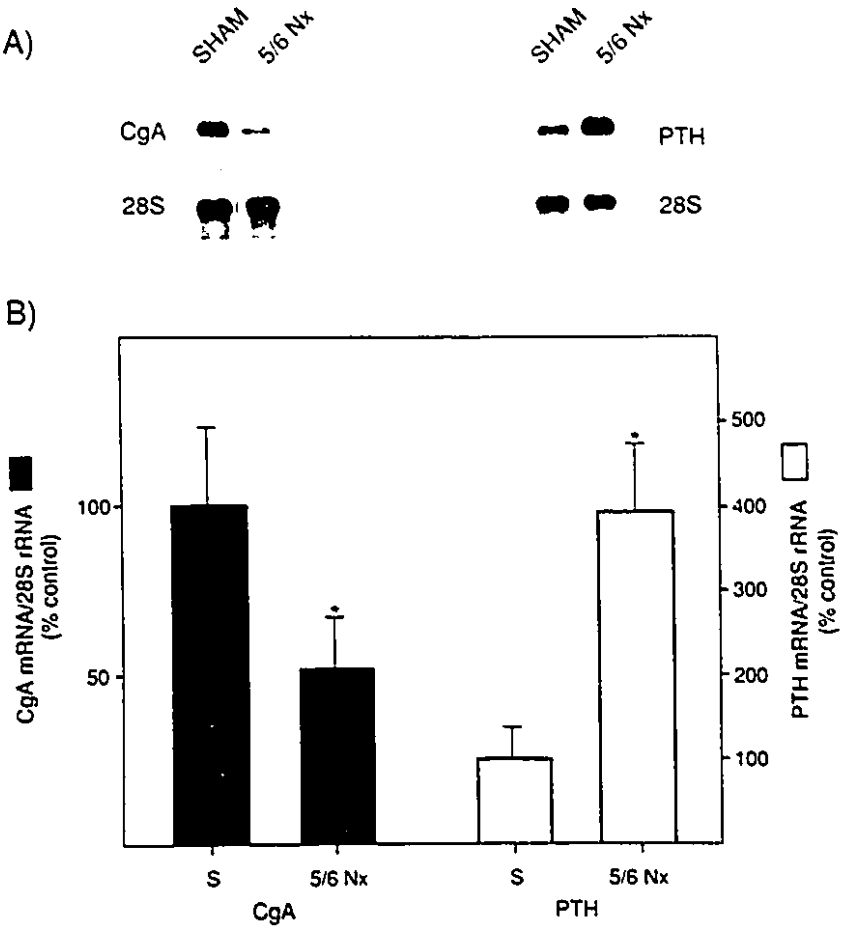

Figure 5. **Parathyroid CgA mRNA levels are decreased in normocalcemic uremic rats fed normal chow.** (A) Autoradiographs of representative Northern blot analysis of parathyroid RNA from rats either sham-operated (SHAM) or 5/6 nephrectomized (5/6 Nx). CgA, PTH and 28S ribosomal RNA were measured as described in Methods. (B) Relative CgA and PTH mRNA levels were assessed by densitometry and related to 28S ribosomal RNA. Each point is the mean ± SEM (n=5-8). Asterisks indicate a significant difference from sham-operated (S) values (*, $p < 0.05$, ** $p < 0.01$). Whereas PTH mRNA levels are increased, those of parathyroid CgA mRNA are decreased in the 5/6 Nx rats.

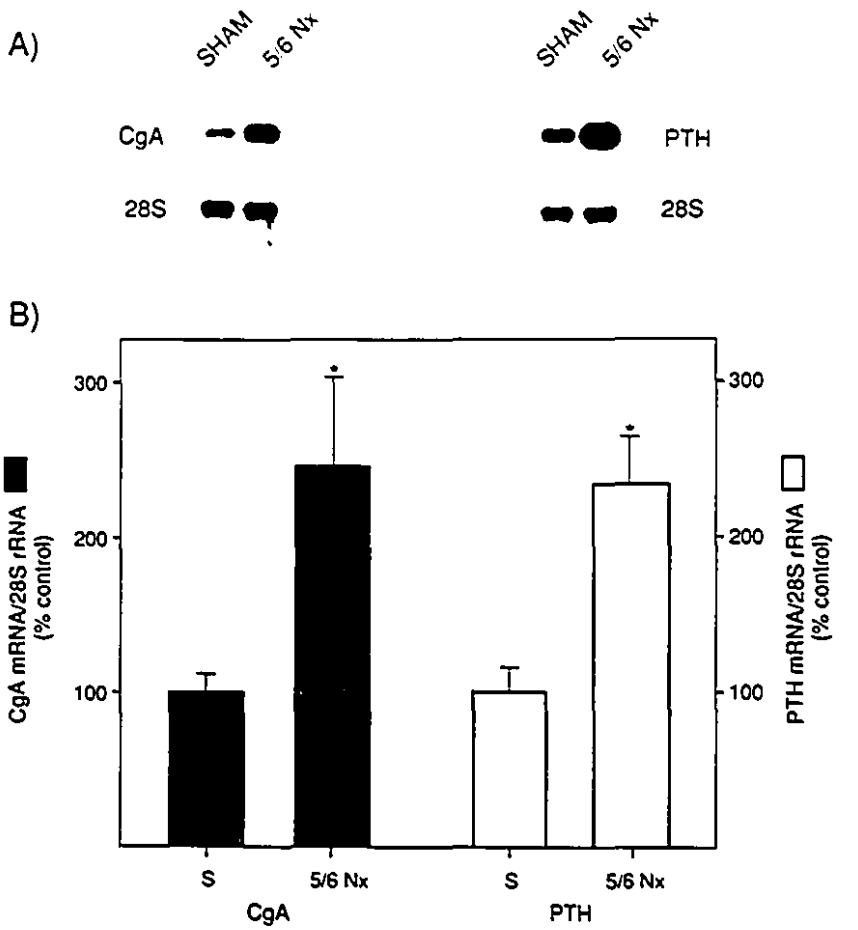

Figure 6. **Parathyroid CgA mRNA levels are increased in the hypocalcemic rats fed a high phosphate diet.** Upper panel (A) shows representative Northern blots of parathyroid RNA from sham-operated and 5/6 nephrectomized (5/6 Nx) rats. Lower panel (B) shows CgA and PTH mRNA expression normalized to that of 28S RNA. Values are mean ± SEM (n=5-8); *, $p < 0.05$, ** $p < 0.01$. Under these dietary conditins, both PTH and CgA mRNA levels are increased.

DISCUSSION

The acidic glycoprotein, CgA, and PTH are the major secretory products of the parathyroid gland (Kemper et al., 1974; Cohn et al., 1982). CgA, the principal member of the granin family of proteins, was originally identified in chromaffin granules of the adrenal medulla and is present in almost all endocrine and neuroendocrine tissues (Winkler et al., 1992). The precise function of this protein, as well as the other members of the granin family, such as chromogranin B (CgB), remains to be fully elucidated. However, recent findings have provided insight as to the intracellular and extracellular roles they might play in controlling cell secretory activity. On the one hand, CgA may act to target or chaperone peptide hormones to granules of the regulated secretory pathway (Parmer et al., 1993); on the other hand, peptides cleaved from the CgA precursor may act in an autocrine or paracrine manner to modulate cell secretion, generally in an inhibitory manner (Hendy et al., 1995).

Expression of neither CgA nor CgB has been examined in the rat parathyroid gland. In the present study, using Northern blot analysis we detected abundant CgA mRNA expression; however a signal for CgB mRNA was not detected suggesting that rat parathyroid gland expresses little, if any, CgB. CgA expression in human parathyroid glands has been demonstrated by Northern blot analysis, immunoblotting, immunohistochemistry and radioimmunoassay (Levine et al.., 1990; Weber et al., 1992). However, investigations of expression of the related granin family member, CgB, in normal human and adenomatous parathyroid tissue have been inconsistent, with most studies reporting negative findings (Hagn et al., 1986; Weiler et al., 1988; Schmid et al., 1989; 1991). The mechanisms governing cell-specific expression of the granins remain to be elucidated, although for the CgA gene we (Mouland et al., 1994; Bevan et al., 1995) and others (Wu et al., 1994; 1995) have shown that sequences in the proximal promoter are important for basal transcription in neuroendocrine cells.

$1,25(OH)_2D_3$ and extracellular calcium are the major regulators of parathyroid gland synthetic and secretory activity (Russell et al., 1983; 1986; Brookman et al., 1986; Cantley et al., 1985; Nygren et al., 1988; Silver et al., 1985; 1986). *In vitro* (Nygren et al., 1988 ; Silver et al., 1986) and *in vivo* (Silver et al., 1986) studies have shown that $1,25(OH)_2D_3$ inhibits PTH release and suppresses PTH mRNA levels by acting at the level of gene transcription (Silver et al., 1985; 1986; Mouland and Hendy, 1991). In contrast to its

inhibitory effect on PTH synthesis, it was shown *in vitro* in primary cultures of bovine parathyroid cells that $1,25(OH)_2D_3$ stimulates CgA mRNA levels and CgA synthesis (Ridgeway et al., 1988; Russell et al., 1990; Mouland and Hendy, 1991). These effects which are dose-and time-dependent are due to a direct effect at the level of gene transcription (Russell et al., 1990; Mouland and Hendy, 1991) rather than changes in mRNA stability (Mouland and Hendy, 1991). In the present study, we show for the first time *in vivo* that parathyroid CgA gene expression is positively regulated in a dose-dependent manner by $1,25(OH)_2D_3$. Importantly, we demonstrate that the parathyroid CgA gene is less responsive acutely than the PTH gene to $1,25(OH)_2D_3$. The molecular mechanisms involved in upregulation and downregulation of gene transcription by the vitamin D sterol are poorly understood, but the present data emphasize the complexity of these processes. The results are consistent with the PTH gene being the more physiologically relevant target for small acute fluctuations in the blood level of $1,25(OH)_2D_3$.

The present studies confirmed that PTH mRNA levels decrease in a dose-dependent fashion in rats injected with $1,25(OH)_2D_3$. The doses of $1,25(OH)_2D_3$ and time frame of the experiments were comparable to those used by other groups (Silver et al., 1986; Shvil et al., 1990; Fukagawa et al., 1991; Brown et al., 1989). For example, Silver *et al.* (1986) observed decreases in PTH mRNA levels in response to injected $1,25(OH)_2D_3$ and concluded that these were specific for changes in serum $1,25(OH)_2D_3$ and independent of changes in serum calcium levels. Here, we observed no change in serum calcium in response to the lowest dose of 10 pmol/100 g, but doses of 50 pmol/100 g and above increased serum calcium and phosphate levels. *In vitro*, Russell *et al.* (1990) found that CgA mRNA was not modulated by low (0.5 mM) or high (2.5 mM) calcium in cultured bovine parathyroid cells studied over a 48 hour period, PTH mRNA was reduced in response to high calcium (Russell et al., 1990). We similarly found a lack of modulation of CgA mRNA by calcium in cultured bovine parathyroid cells (Mouland and Hendy, 1991). In contrast, Zhang *et al.* (1993) found that porcine parathyroid cells cultured in high calcium (3 mM) expressed lower CgA mRNA levels than those cultured in low calcium (0.5 mM). The apparent discrepancies between these studies may relate to a different species responsiveness to calcium, or to some aspect of the culture system used. For example, the concentration of serum used in the medium may profoundly alter the expression of the parathyroid calcium-sensing receptor (Mithal et al., 1995), and the relative concentration of

glucocorticoid in the medium may modulate the relative sensitivity of the regulation of parathyroid CgA mRNA by calcium (Zhang et al., 1993). Although some uncertainty remains as to the precise relationship between extracellular calcium and parathyroid CgA mRNA levels *in vitro* it is likely the elevations in plasma calcium that accompany increases in $1,25(OH)_2D_3$ after administration of the sterol *in vivo* would tend to dampen the increase in CgA mRNA level rather than stimulate it.

The reduced circulating level of $1,25(OH)_2D_3$ is an important factor in the development of secondary hyperparathyroidism associated with chronic renal failure (Lopez-Hilker et al., 1986; Bover et al., 1994; Shvil et al., 1990; Reichel et al., 1993). Having observed regulation of CgA gene expression by this sterol in normal rats, it was then of interest to examine whether CgA gene expression was altered in a rat model of secondary hyperparathyroidism in which circulating $1,25(OH)_2D_3$ levels were reduced, but calcium levels were unaltered. In these uremic rats, PTH mRNA levels were increased four-fold, serum PTH levels were almost double those found in sham-operated rats, and CgA gene expression was reduced by approximately 50%.

Nephrectomized rats fed a high phosporus diet had reduced serum levels of both $1,25(OH)_2D_3$ and calcium. In this situation, the parathyroid gland content of both CgA and PTH mRNA was increased, indicating that the stimulatory effect on CgA mRNA of hypocalcemia predominates over the inhibitory effect of reduced circulating levels of $1,25(OH)_2D_3$ (Table 4). A similar effect had been noted previously with respect to the PTH mRNA levels of rats fed a low calcium diet (Naveh-Many et al., 1990). These hypocalcemic animals had increased PTH mRNA levels despite having elevated serum $1,25(OH)_2D_3$ levels.

In summary, we have shown that *in vivo* increased serum $1,25(OH)_2D_3$ concentrations stimulate, and decreased concentrations inhibit parathyroid CgA gene expression. In mild renal failure with reduced serum $1,25(OH)_2D_3$ levels, but normocalcemia, parathyroid CgA mRNA levels are reduced, whereas those of PTH mRNA are increased. In contrast, in mild renal failure with reduced serum $1,25(OH)_2D_3$ and calcium levels, parathyroid CgA mRNA and PTH levels are both increased. Thus, the calcium status of the animal modulates the responsiveness of parathyroid CgA levels to the circulating vitamin D metabolite.

PART 3:

GENERAL DISCUSSION

Synthesis and secretion of parathyroid CgA has been shown to be influenced by the main regulators of parathyroid cell function; calcium and $1,25(OH)_2D_3$. While controversy surrounds the *in vitro* regulation of CgA synthesis by calcium (Russell et al., 1990; Mouland and Hendy., 1991; Zhang et al., 1993), $1,25(OH)_2D_3$ clearly increases both the synthesis and secretion of CgA in cultured parathyroid cells by acting at the transcriptional level of the biosynthetic pathway (Russell et al., 1990; Mouland and Hendy, 1991). *In vitro* experiments provide a more controlled environment to examine the specific effects of various agents. On the other hand, because *in vivo* models represent a more physiological setting, we analyzed the regulation of $1,25(OH)_2D_3$ on CgA synthesis in the normal rat. The results obtained are consistent with those observed *in vitro*; a dose-dependent increase in CgA mRNA levels was observed upon administration of $1,25(OH)_2D_3$. Regarding the smallest dose of 10 pmol, although serum $1,25(OH)_2D_3$ levels were not different from control at 24 hours after the second injection, it has been demonstrated that such a dose causes a rapid increase in circulating $1,25(OH)_2D_3$ levels within 12 hours and that these return to baseline values within 24 hours (Reichel et al., 1993). Also of interest in these acute studies was the finding that CgA expression is less sensitive to the sterol than PTH however, in the chronic studies, discussed below, CgA mRNA levels were responsive to modest changes in circulating $1,25(OH)_2D_3$ levels.

Having established the regulation of CgA by $1,25(OH)_2D_3$ in the intact rat, we then extended our analysis of parathyroid CgA gene expression to an experimental model of mild chronic renal failure in which levels of $1,25(OH)_2D_3$ are reduced and are believed to be important in the pathogenesis of 2°HPT. The induction of a mild type of renal failure in a uremic rat model in combination with the feeding of a normal diet provided an opportunity to analyze the effects of reduced circulating $1,25(OH)_2D_3$ levels *per se* on parathyroid CgA gene expression. Nephrectomized rats had a 50% reduction in CgA gene expression and a 4-fold increase in PTH mRNA levels. As in the acute experiments, $1,25(OH)_2D_3$ oppositely regulates CgA and PTH gene expression, however these experiments demonstrate the effects of a reduction in $1,25(OH)_2D_3$ levels. Since CgA is reduced by only one-half and PTH is increased 4-fold, these chronic studies also suggest that CgA gene expression is less responsive to the sterol than is PTH.

As the precise role that CgA plays in the parathyroid cell is unclear, the

consequences of altered parathyroid CgA synthesis is not known. Intracellularly, it is suggested that by its ability to aggregate with some types of molecule but not others in the *trans*-Golgi network, CgA directs the sorting and packaging of peptide hormone and neurotransmitters to granules of the regulated secretory pathway in neuroendocrine cells (Parmer et al., 1993). Indirect evidence for this comes from *in vitro* studies showing that, under low pH and high calcium conditions, like those found in the *trans*-Golgi network adjacent to budding secretory granules, CgA coaggregates with a peptide hormone such as PTH, but excludes constitutively secreted proteins such as serum albumin (Gorr et al., 1989). Extracellularly, several CgA-derived peptide such as ß-granin [CgA-(1-113)] (Drees et al., 1991), synthetic CgA-(1-40) (Russell et al., 1994), pancreastatin [porcine CgA-(240-288)] (Fasciotto et al., 1989), and parastatin [porcine CgA-(347-419)] (Fasciotto et al., 1993) have been shown to inhibit the low calcium-stimulated secretion from cultured parathyroid cells. Addition of CgA antibodies to the medium of cultured parathyroid cells leads to increased PTH release from the cells (Fasciotto et al., 1990). The mechanism of action of CgA-peptides on parathyroid cells is unknown, although it is assumed to be receptor-mediated. It is not known whether *in vivo* CgA acts in a predominantly intracellular or extracellular manner in the parathyroid cell. However, it should be noted that little processing of CgA to peptides occurs within the parathyroid cell (Fasciotto et al., 1992; Drees et al., 1992). Therefore, if CgA peptides are active on parathyroid cells *in vivo* they are probably derived extracellularly or are released from neighbouring nonparathyroid cells.

The data obtained in the present study support the hypothesis that CgA plays a predominantly intracellular role in the secretory process in the parathyroid. In the normocalcemic uremic rat model, in the face of reduced $1,25(OH)_2D_3$ levels, PTH and CgA synthesis, as represented by alterations in their mRNA levels, increase four-fold and decrease by 50%, respectively. The parathyroid secretory response is represented by the measurement of circulating intact PTH. Circulating mid-region and carboxyl-terminal PTH fragments which increase in the circulation as a result of decreased clearance of these moieties in renal failure (Martin et al., 1979) are not measured by this assay. The amount of intact PTH released by the gland doubles, whereas PTH synthesis is increased four-fold (Fig. 7). This two-fold difference in synthesis versus release could be explained by the 50% decrease in CgA synthesis if the predominant role of CgA is intracellular to promote the appropriate trafficking of PTH in the regulated secretory pathway. Put another way, if the

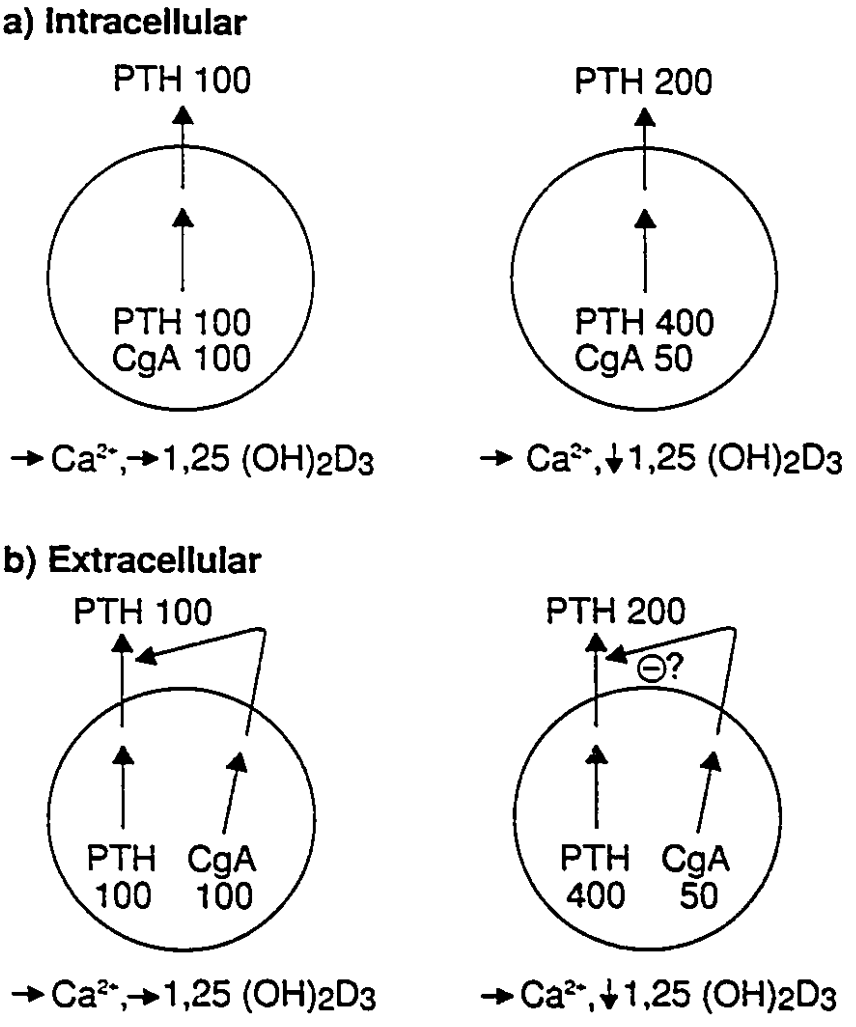

Figure 7. **Chromogranin A plays a predominantly intracellular role in the secretory process in the parathyroid.** In the model shown, PTH and CgA synthesis (represented by their respective mRNA levels) and the parathyroid secretory response (represented by circulating intact PTH levels) are compared between sham-operated (control) rats (on the left) and 5/6 Nx (uremic) rats fed a normal chow (on the right). Control values are set at 100%. The new steady-state values achieved in the uremic rats are: PTH synthesis, 400%; CgA synthesis, 50% and PTH secreted, 200% relative to control. Panel a) the data are consistent with CgA playing an essential role intracellularly to guide PTH through the regulated secretory pathway. Panel b) the data are inconsistent with CgA (or CgA-derived peptides) acting extracellularly to inhibit PTH secretion.

predominant role of parathyroid CgA is to suppress PTH release via extracellular actions of CgA-derived peptides (Fig. 7), PTH secretion relative to synthesis would increase in the face of the decrease in parathyroid CgA observed in the uremic rats. This is clearly not the case.

In uremia there is progressive retention of CgA immunoreactive fragments which is the result of renal dysfunction alone, rather than hyperparathyroidism (Hsiao et al., 1990). The parathyroid makes a minor contribution to the total circulating pool of CgA relative to other endocrine glands and sympathetic neurons (Takiyyuddin et al., 1990). If circulating CgA fragments are biologically active, an increase in their concentration in chronic renal failure may affect secretion from endocrine cells. However, *in vitro* a CgA peptide concentration of approximately 10^{-8} M is required for inhibition of parathyroid cell secretory activity (Hendy et al., 1995; Russell et al., 1994; Fasciotto et al., 1993), whereas the circulating concentration of CgA is 10^{-10} M (Hsiao et al., 1990; O'Connor et al., 1990; Nanes et al., 1989). Therefore, a substantial increase in circulating CgA levels would be required before they have an impact on parathyroid cell secretory function. Hsiao *et al* (1990) presented circulating CgA values in human subjects stratified by degree of renal insufficiency. In subjects with mid-range renal disease in whom serum creatinine and intact PTH were four-fold normal, serum CgA levels were five-fold those of normal subjects. In the uremic rats described here, serum creatinine and intact PTH levels were only elevated two-fold. By extrapolation from the data in humans (Hsiao et al., 1990), it can be estimated that in the uremic rats CgA levels would be increased less than five-fold. Therefore, circulating CgA levels are unlikely to modulate parathyroid gland secretion even in mild renal failure.

Further insight into the *in vivo* regulation of parathyroid CgA was provided by the experiments in which nephrectomized rats were fed a high phosphorus diet. The changes in parathyroid CgA mRNA levels relative to the change in PTH mRNA levels and PTH secretion again provide further support for CgA playing a predominantly intracellular role in the parathyroid. PTH gene expression (synthesis) and circulating intact PTH levels (secretion) both increase by 2.5 fold as does parathyroid CgA synthesis. If CgA was acting in a predominantly extracellular fashion, one would have expected PTH secretion to be decreased relative to PTH gene expression. This is not the case, and the parallel increases in CgA and PTH gene expression could be seen as a consequence of the need for enhanced packaging and secretion of PTH which is mediated by CgA. The data support the notion

that *in vivo* CgA plays a predominantly intracellular role in modulating parathyroid cell secretory activity.

Our studies demonstrate that $1,25(OH)_2D_3$ regulates CgA mRNA *in vivo* in the parathyroid. It is clear that the CgA gene is responsive to $1,25(OH)_2D_3$ and while its cloning has led to the partial characterization of cis-acting response elements in the 5' promoter region, a functional VDRE has yet to be identified. Certain studies could be performed in order to demonstrate binding of a DNA sequence to a respective receptor. These include DNA footprinting assays and gel electrophoresis mobility-shift assays. Functional studies would involve placing the DNA sequence containing the putative VDRE upstream of a reporter gene such as chloramphenicol acetyltransferase and transfecting these constructs into cells. It would also be of interest to explore the tissue-specific response of $1,25(OH)_2D_3$ on CgA gene expression, in other words, to analyze whether CgA gene expression in other tissues besides the parathyroids (such as the pituitary and adrenal) is regulated by $1,25(OH)_2D_3$. These studies could be extended to determine whether a correlation exists between responsiveness and VDR expression.

Because synthesis of a protein is not always directly proportional to the amount of its mRNA present, it would be worth pursuing the relationship between measured CgA mRNA levels and protein levels inside the parathyroid gland as well as in the circulation. While Western blot analysis and immunohistochemistry would be useful means to assess relative changes in protein levels inside the parathyroid glands, a radioimmunoassay is necessary to measure serum CgA levels. Furthermore, to test whether circulating CgA-derived fragments can effect parathyroid gland secretory activity it would be of interest to infuse synthetic fragments at various concentrations and analyze expression and secretion of parathyroid CgA and PTH.

While our studies have focused on CgA gene expression with relation to changes in circulating calcium and $1,25(OH)_2D_3$, speculation may arise as to whether phosphate plays a role in modulating CgA and PTH. Recent findings demonstrate that phosphate indeed modulates PTH gene expression (Kilav et al., 1995), however, the mechanisms by which this ion acts are unknown. It may, for instance, be functioning via an indirect manner by influencing extracellular and/or intracellular calcium levels. Further experiments will be required to determine these precise mechanisms.

Our studies demonstrate that calcium plays a role *in vivo* in regulating parathyroid

CgA gene expression, however, because the circulating levels of this ion and $1,25(OH)_2D_3$ are inter-related, it is often difficult to evaluate the effects of calcium *per se*. Experimentally, rats could be injected with calcium (in the form of $CaCl_2$) to increase circulating levels of this ion. Likewise, a chelator (such as EDTA) or calcitonin could be used to reduce circulating levels of calcium. Analyzing changes in gene expression under various calcium concentrations but normal circulating levels of $1,25(OH)_2D_3$ would provide further evidence for a role of calcium in CgA gene regulation.

The cloning of the PTH/PTHrP receptor (Abou-Samra et al., 1992; Pausova et al., 1994) has led groups to investigate whether altered expression might be a cause of the PTH resistance observed in 2°HPT. We (unpublished results; Fig. 8) and others (Ureña et al., 1994b; 1995) have observed a reduction in PTH/PTHrP receptor mRNA in the remnant kidney in uremic rats with experimental 2°HPT which have normal circulating levels of calcium and phophate, decreased $1,25(OH)_2D_3$ concentrations and increased PTH levels. However, we find no change (Fig. 10) in PTH receptor expression in the femur of nephrectomized animals whereas Ureña and associates (1995) observed a decrease in expression in femoral head tissue. This descrepancy may be due to a difference in expression which is dependent on the anatomical segment analyzed. With regards to non-classic tissues, some have found decreases in receptor expression in liver (Tian et al., 1994) and heart (Smogorzewski et al., 1995) of uremic rats, whereas others have found no change in the former tissue (Ureña et al., 1994b; 1995).

Since calcium levels are normal in our model of CRF, hypocalcemia is unlikely to be the cause of down-regulation in expression. On the other hand, *in vitro* experiments demonstrate that PTH decreases expression of the PTH/PTHrP receptor in UMR 106-01 osteoblastic-like cells (Gonzales and Martin, 1995). This suggests that the elevated PTH levels observed in 2°HPT might be an important factor in altering expression. However, when normal rats are subjected to thyroparathyroidectomy ten days before the 5/6 nephrectomy, PTH/PTHrP receptor expression is nevertheless decreased (five weeks after nephrectomy) in the presence of reduced PTH levels (Ureña et al., 1995), indicating that an increase in plasma PTH is not solely responsible for renal PTH receptor down-regulation during chronic renal failure. In contrast to the results obtained by Ureña et al. (1995), Tian and associates (1994) observed an improvement (kidney) and complete normalization (heart

and liver) in PTH/PTHrP receptor mRNA levels in normocalcemic chronic renal failure rats subjected to parathyroidectomy seven days prior ro 5/6 nephrectomy (six weeks duration). Because treatment of uremic rats with verapamil, a calcium channel blocker, normalizes cytosolic calcium levels in cardiac myocytes as well as receptor mRNA levels (Smogorzewski et al., 1995), the authors suggest that PTH-induced increases in intracellular calcium is a potential cause of the down-regulation in receptor expression observed in CRF.

A reduction in $1,25(OH)_2D_3$ levels should also be considered as a potential factor in the altered expression of PTH/PTHrP receptor in 2°HPT. *In vitro* studies suggest that $1,25(OH)_2D_3$ does regulate expression of this receptor. For example, calcitriol increases mRNA levels of the receptor in primary cultures of mouse renal distal tubular cells (Sneddon et al., 1993) but down regulates expression in UMR 106-01 cells (Gonzales and Martin, 1995). However, our preliminary *in vivo* results demonstate that administration of supraphysiological doses of $1,25(OH)_2D_3$ do not markedly alter receptor mRNA levels in rat femur and kidney (Figs. 9 and 10). Further studies will be required to elucidate whether $1,25(OH)_2D_3$ *per se* regulates PTH receptor expression in these organs, especially bone, since this negative result observed upon administration to normal rats may be caused by analyzing the entire bone versus various anatomical segments of the femur thus potentially diluting out any changes in expression in a specific small subset of cells relative to the total number of PTHR-expressing cells. In addition, thyroparathyroidectomy to normal rats leads to reduced levels of the sterol but receptor expression remains unaltered (Ureña et al., 1995). In view of what is presently known about alterations in receptor expression, it would be of interest to inject chronic renal failure rats with $1,25(OH)_2D_3$ and evaluate if these are capable of normalizing receptor expression.

A recent study (Turner et al., 1995) has provided further insight into tissue-specific regulation of the PTH/PTHrP receptor. Although it was confirmed that rats with low circulating levels of calcium and $1,25(OH)_2D_3$ (induced by dietary means) have a decrease in renal membrane adenylyl cyclase activity, there was also, in contrast to the findings of Ureña et al. (1994b) and Tian et al. (1994), a concomitant increase in PTH/PTHrP receptor mRNA content in the kidney and the epi/metaphysis, but not the diaphysis. Although the changes in serum PTH and $1,25(OH)_2D_3$ concentrations were similar in all studies, these descrepancies in expression may be due to factors intrinsic to uremia which would evidentiy not be present in the dietary model. These may include substances such as cytokines and

angiotensin II which are increased in chronic renal failure (Herbelin et al., 1991; Rosenberg et al., 1994) and have been shown to decrease PTH/PTHrP receptor expression *in vitro* (Katz et al., 1992; Okano et al., 1994). The lack of correlation between receptor function and expression points to post-transcriptional defects as the possible cause of the renal resistance. Partial normalization of expression in bone was achieved by vitamin D supplementation or a high calcium diet, whereas only the former completely normalized mRNA levels in the kidney.

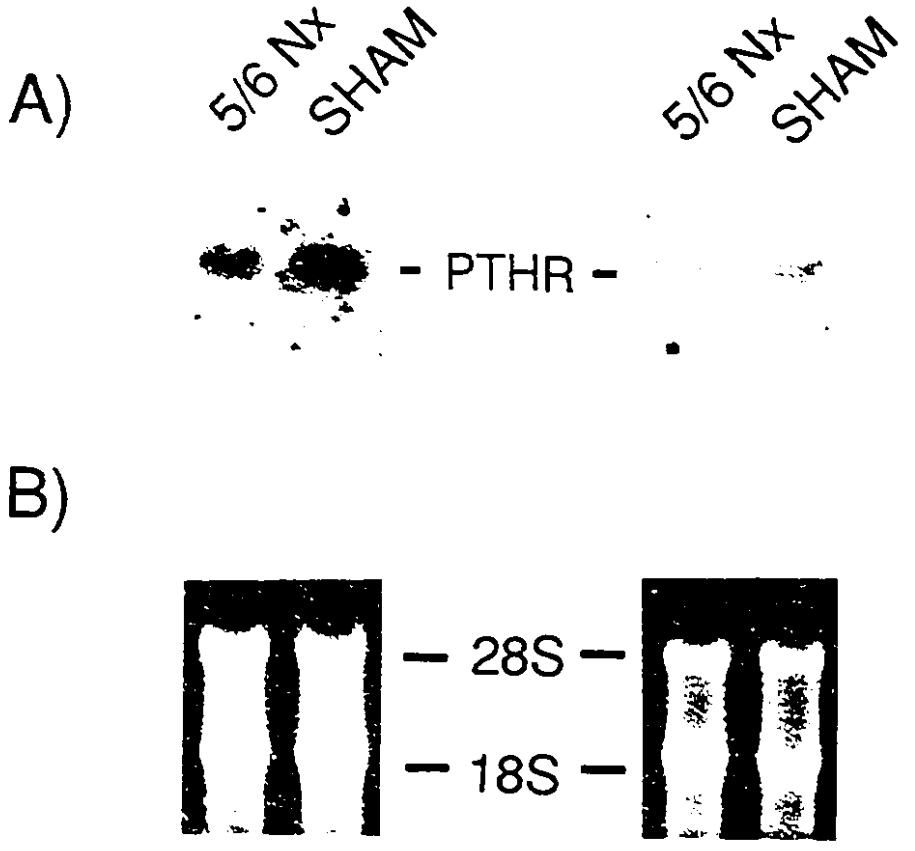

Figure 8. **PTH/PTHrP receptor mRNA levels are decreased in kidney of 5/6 nephrectomized rats.** Blots were probed using a cDNA specific for rat PTH/PTHrP receptor mRNA. A) Autoradiograph of representative Northern blots of kidney RNA obtained from either sham-operated (SHAM) or 5/6 nehrectomized (5/6 Nx) rats. Expression for receptor in uremic kidneys is clearly decreased as compared to controls. B) Relative RNA quantitiy and quality was monitored by ethidium bromide staining of 28S and 18S ribosomal RNA.

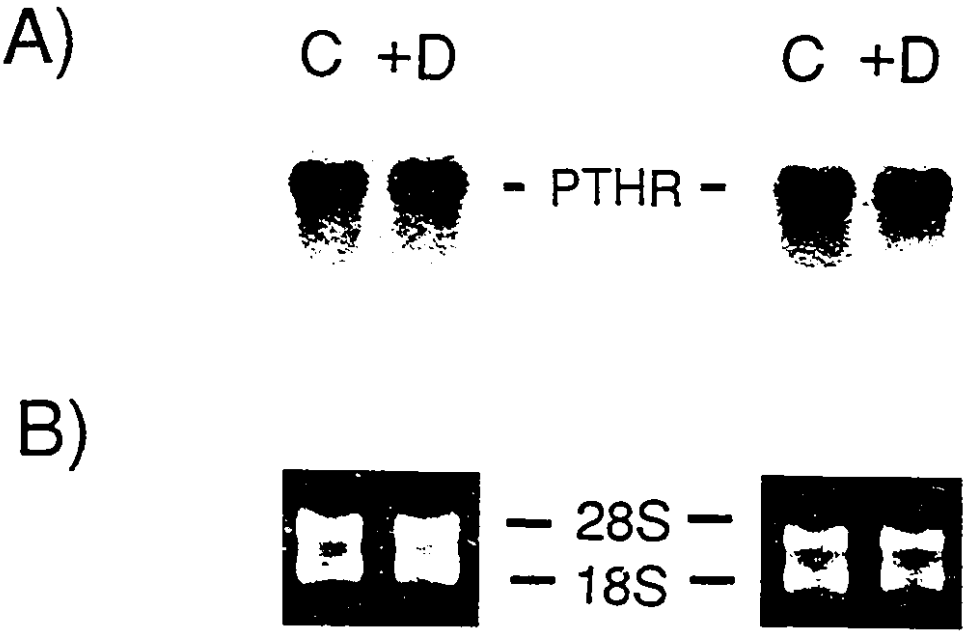

Figure 9. **PTH/PTHrP receptor mRNA levels are unchanged in kidney of rats administered $1,25(OH)_2D_3$.** Rats were administered 500 pmol of $1,25(OH)_2D_3$ at 48 and 24 hours prior to sacrifice. A) Autoradiographs of representative Northern blots containing kidney RNA obtained from control rats (C) or those injected with $1,25(OH)_2D_3$ (+D). The sterol does not seem to regulate renal expression of the receptor. B) Relative RNA quantitiy and quality was monitored by ethidium bromide staining of 28S and 18S ribosomal RNA.

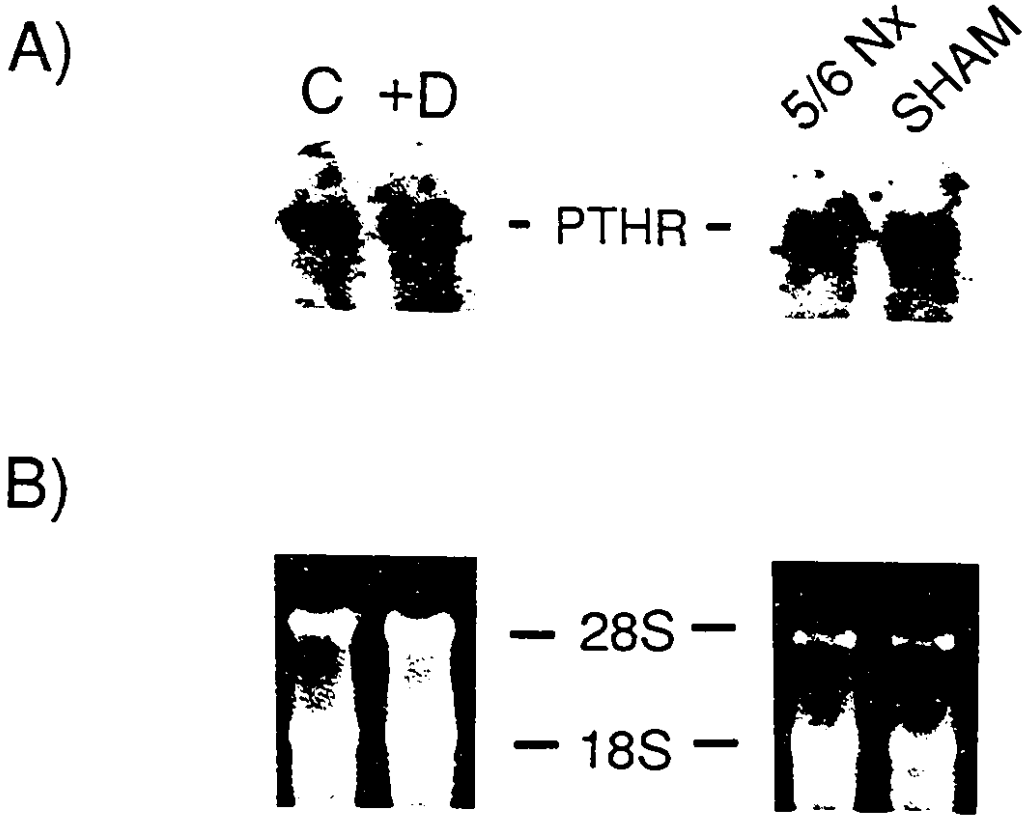

Figure 10. **PTH/PTHrP receptor mRNA levels are unchanged in femur of rats administered 1,25(OH)$_2$D$_3$ or made deficient by subjecting them to 5/6 nephrectomy.** A) Northern blot analysis was conducted on RNA obtained from rat femur. One group of rats was administered either vehicle (C) or 500 pmol of 1,25(OH)$_2$D$_3$ (+D) at 48 and 24 hours prior to sacrifice (left). Another group was subjected to either sham operation (SHAM) or 5/6 nephrectomy (5/6 Nx) (right). Neither rats with increased or decreased circulating levels of 1,25(OH)$_2$D$_3$ had changes in femoral expression of the PTH/PTHrP receptor. B) Relative RNA quantitiy and quality was monitored by ethidium bromide staining of 28S and 18S ribosomal RNA.

PART 4:

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