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U N I V E R S I T Y O F A L B E R T A

PARATHYROID HORMONE (PTH) AND PTH-LIKE PEPTIDES IN NEURAL TISSUES

BY

ROBERT A. FRASER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **DOCTOR OF PHILOSOPHY**

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

SPRING, 1991

ISBN 0-315-66769-9

UNIVERSITY OF ALBERTA

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TITLE OF THESIS: PARATHYROID HORMONE (PTH) AND PTH-LIKE PEPTIDES IN NEURAL TISSUES

DEGREE: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: SPRING, 1991

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C. BENISHIN

November 8, 1990

To my mother, my father and my sisters with love.

A B S T R A C T

The presence of parathyroid hormone (PTH) and PTH-like peptides (stanniocalcin and PTH-related peptide, PTHrp) in hypothalamic and extra-hypothalamic tissue has been determined in vertebrate species by immunoreactive (IR), chromatographic and molecular biological techniques. Radioimmunoassays (RIAs) using antisera for PTH-(53-64), -(48-53) and-(1-34) detected IR PTH in the brains and pituitary glands of piscine, amphibian, reptilian, avian and mammalian species. The IR PTH from rat hypothalamic and pituitary extracts co-eluted from a reverse phase (rp) high performance liquid chromatography (HPLC) column with authentic human PTH. Messenger ribonucleic acid (mRNA) from rat hypothalamus contained a moeity capable of cross hybridizing with a specific complementary RNA (cRNA) probe in a Northern blot analysis and with two specific PTH 5' and 3' oligonucleotide primers in a polymerase chain reaction (PCR) analysis. In both studies, hybridizing bands co-migrated through electrophoretic gels with PTH mRNA from the rat parathyroid gland. Using a PTH cRNA radiolabelled probe, PTH mRNA was localized by *in situ* hybridization in the paraventricular and supraoptic nuclei of the rat hypothalamus.

Immunoreactive stanniocalcin, PTHrp and PTH were identified in salmon pituitary extracts and found to co-elute from a rpHPLC column with their respective authentic peptides. The IR stanniocalcin in platyfish was located in nerve fibres in the neuro-pars intermedia and in cell bodies in the pars magnocellularis and pars parvocellularis of the preoptic nuclei, similar to previous reports of IR PTH distribution in the fish central nervous system (CNS).

Furthermore, immunoreactivity, specific for PTHrp, was demonstrated in extracts of the rat hypothalamus and pituitary gland. A mRNA from rat hypothalamus of ~1.8 kb, hybridized with a radiolabelled PTHrp cDNA probe and was shown to co-migrate with known PTHrp mRNA from cultured parathyroid cells in a Northern blot.

These results describe novel neuropeptidergic systems located in similar regions of the brain for three hormones that participate in peripheral calcium homeostasis and suggest evolutionary and functional relationships for these peptides in neural physiology.

A C K N O W L E D G E M E N T S

For the completion of this work I am indebted to numerous individuals and agencies. I am especially indebted to my supervisor, Dr. Steve Harvey for his expertise, advice, training and patient supervision. I would like to give special thanks to Dr. Hank Kronenberg, Dr. Marie Demay, Dr. Jeff Zajac, Dr. Freda Miller, Mr. Philip Barker, Dr. Peter Pang, Dr. Toyoji Kaneko and Mrs. Teresa Labedz and Mr. Dan Fackre for their expert advice and training in molecular biological, immunoreactive studies and chromatographic studies. This work was made possible by the generous gifts of rPTH and hPTHrp cDNA, hPTHrp and bPTH antibodies, teleocalcin and hypocalcin peptides and hypophysial portal and jugular sheep serum from Drs. G. Heinrich, J. Zajac, T.J. Martin, C. Cooper, D.H. Copp, S.E. Wendelaar Bonga and F.J. Karsch, respectively. Funding for these studies was provided by the Medical Research Council of Canada and the National Science and Engineering Research Council, the Alberta Heritage Foundation for Medical Research and the US National Institute of Health (DK 11794). I would also like to thank Ms. J. Gatzke for typing the thesis.

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LIST OF ABBREVIATIONS

ABC:	avidin-biotin complex
ACTH:	adrenocorticotropic hormone
ADP:	atrial natriuretic peptide
APUD:	high amine content, amine precursor uptake and decarboxylation
ATPase:	adenosine triphosphatase
BSA:	bovine serum albumin
cAMP:	cyclic adenosine monophosphate
CCK:	cholecystokinin
cDNA:	complementary deoxyribonucleic acid
CGRP:	calcitonin gene-related product
CNS:	central nervous system
COOH-terminal:	carboxyl-terminal
CRE:	cAMP response element
CREB:	CRE binding protein
CRF:	corticotrophic releasing factor
cRNA:	complementary RNA
CS:	corpuscles of Stannius
CTP:	cytidine triphosphate
DAG:	diacyl glycerol
dATP:	deoxyadenosine triphosphate
ELISA:	enzyme-linked immunosorbant assay
FSH:	follicle stimulating hormone
GH:	growth hormone

HHM:	humoral hypercalcemia of malignancy
HPLC:	high performance liquid chromatography
ICC:	immunocytochemistry
IGFI and -II:	insulin like growth factor I and II
IHC:	immunohistochemistry
IP_3:	inositol triphosphate
IR:	immunoreactive
IVS:	intervening sequence
LH:	leuteinizing hormone
LHRH:	leuteinizing hormone releasing hormone
M-MLV:	Moloney Murine Leukemia Virus
mRNA:	messenger ribonucleic acid
MSH:	melanotropin stimulating hormone
NMR:	nuclear magnetic resonance
NPO:	nucleus preopticus
N-terminal:	amino-terminal
OK cells:	oppossum kidney cells
Opt:	optic tract
PaV:	paraventricular
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PCS:	parathyrin from the CS
PD:	pars distailis
PI:	pars intermedia
PHI:	polypeptide with amino terminal histidine and carboxyl-terminal isoleucine amide

pm:	pars magnocellularis
Poly A+:	polyadenylated
POMC:	pro-opiomelanocortin
pp:	pars parvocellularis
PTH:	parathyroid hormone
PTHrp:	PTH related peptide
PTX:	parathyroidectomy
PV:	periventricular
PvLM:	paraventricular lateral magnocellular
RADH I, II, III:	rabbit anti-Dutch hypocalcin I, II, III
RIA:	radioimmunoassay
rp:	reverse phase
SO:	supraoptic
STX:	stannieclomized
Taq DNA polymerase:	Thermus aquaticus DNA polymerase
TFA:	trifluoracetic acid
3V:	third ventricle
TRH:	thyrotropin releasing hormone
TSH:	thyrotropin
VIP:	vasoactive intestinal peptide

CHAPTER I

GENERAL INTRODUCTION

The name and popularity of a peptide may be deleterious
Kastin et al., 1987

NEUROPEPTIDES: THE PARANEURAL SYSTEM

INTRODUCTION

Communication within and between cells represents a major physiological function of all living uni- and multi-cellular organisms. Traditionally, messages between cells were thought to be mediated by chemical messengers of neural or endocrine origin; synaptic transmission was mediated solely by "classical" neurotransmitters (the monoamines, amino acids and acelylcholine) and endocrine transmission was mediated solely by "classical" hormones (peptide, thyroid and steroid hormones). This traditional concept was challenged by Scharrer and Scharrer (1940) who proposed that a neuron could release peptides into the bloodstream for actions as "hormones". Later, it was demonstrated that neural projections from the paraventricular region of the hypothalamus into the posterior pituitary of rats (Bergeman, 1960) could secrete vasopressin and oxytocin into the bloodstream. It has also been shown that the cells synthesizing these hormones have central projections as well, and that they may have functions accordingly (Kreiger, 1984). It is now well established that peptides, like conventional neurotransmitters, generate rapid effects post-synaptically (Iversen *et al.*, 1980) and modulate pre- and postsynaptic membrane potentials, by altering ion conductance through ligand-operated channels (Snyder, 1980). Furthermore, improvements in peptide technology have made possible

the detection and isolation of peptides in trace amounts. Today, the wide distribution and numerous functions of peptides in central and peripheral tissues is considered the rule rather than the exception (Table I-1.)

DETECTION, ISOLATION AND CHARACTERIZATION OF PEPTIDES

In order to study the physiology of a peptide and its receptor, it is necessary to identify and purify the peptide, its messenger ribonucleic acid (mRNA) or its gene. The detection and isolation of biologically active peptides in ectopic and nerve tissues has been accomplished through a variety of approaches based on the following:

Biological Activity. Peptides can be identified using specific, reproducible assays to monitor their biological activities. The purification of all peptides requires evaluation by bioassay. Peptides may be classified as "families" based on similar actions in bioassays (*e.g.* VIP, Said, 1984).

Immunoreactivity. The antigenicity of peptides is a useful characteristic for determining presence and concentrations of peptides in a tissue or tissue extract. The raising of specific monoclonal antibodies or polyclonal antisera for radioimmunoassays (RIAs) and/or immunocytochemistry (ICC) have been useful for first recognizing peptides in brain (Hokfelt *et al.*, 1980; 1984; Lundberg and Hokfelt, 1983; Palkovitis, 1984). The use of multiple antisera with specificities for different portions of the peptide sequence improves the dependability of initial immunoreactive detection. Recent immunometric assays have even greater sensitivity,

specficity and precision than RIAs and are free from non-specific serum effects (Segre, 1990). The use of high- and weak-affinity chromatography (Zopf and Ohlson, 1990) has also been useful in purifying antigens from tissue extracts.

Physiochemical Characteristics. Each peptide has distinct physiochemical properties that faciliate its purification. The reproducibility, efficiency and resolution of chromatographic separation has been greatly improved by the advent of high performance liquid chromatography (HPLC). Electrophoretic separation has been improved by two dimensional and pulsed-field gel electrophoresis. Detection of peptides bearing carboxyl-terminal amides, peptides of biological importance, by enzymatic methods, has led to the isolation of the novel peptides PHI (*p*olypeptide with amino-terminal *h*istidine and carboxy-terminal *i*soleucine amide) and neuropeptide Y (Tatemoto *et al.*, 1982).

Ligand Receptor Binding. The identification of the endogenous opioid peptides was triggered by the pharmacological demonstration that exogenously administered pharmaceuticals (e.g. benzodiazapine) specifically bound to endogenous receptors in brain extracts (Snyder and Childers, 1979). The opioid peptides were subsequently isolated and characterized based on their capability of binding specifically to these brain membrane protein preparations (Guidotti *et al.*, 1983). Receptor binding assays are useful in locating and characterizing peptide activity.

Molecular Biology. Molecular biological techniques have been extremely useful in the isolation and characterization of peptides and their precursor structures and for peptide synthesis. For instance, determination of the complementary deoxyribonucleic acid (cDNA) structure of pro-opiomelanocortin (POMC) and of pro-enkephalin A and B revealed the previously unknown peptides melanotropin stimulating hormone (MSH) and multiple forms of enkephalin, respectively (Hale and Rees, 1989). As well, the sequencing of the calcitonin gene led to the recognition of an alternatively spliced transcript, calcitonin gene related product (CGRP) (Goodman and Iversen, 1986). Screening of cDNA libraries using antibodies or cDNA probes has greatly accelerated determination of mRNA nucleotide and translated amino acid sequences. The synthesis of complementary RNA (cRNA) probes (Melton *et al.*, 1986) for use in Northern blot and *in situ* hybridization and the polymerase chain reaction (PCR) (Saiki *et al.*, 1985) have further enhanced the specificity and sensitivity of mRNA detection within tissues. PCR has also been used to rapidly clone and sequence novel peptide mRNA and genetic sequences. Genetic sequences have been studied to determine the function and synthesis of peptides in single cell (transfected) and whole animal (transgenic) models.

THE DIVERSE ENDOCRINE SYSTEM

Diverse Peptide Function

Peptides can be localized histologically, within endocrine cells of peripheral endocrine glands and the gastrointestinal tract and in

nerve cells within the central and peripheral nervous systems. In these locations peptides may have endocrine, paracrine, autocrine and intracrine roles (Fig. I-1).

Peptides of the endocrine system are secreted into the circulation for actions on a distant target tissue (Fig. 1a). Classical examples of peptides of this system include insulin, glucagon, gastrin and growth hormone. Peptides of the type originating from a nerve cell would be neuroendocrine peptides (e.g. oxytocin and vasopressin)(Fig. I-1).

Paracrine and autocrine functions represent local actions of peptides released from their endocrine cells. In paracrine systems the secreted product reaches neighboring cells by extracellular fluid within the interstitial space or via intracellular gap junctions (e.g. peptides of reproductive organs and the gastrointestinal tract) (Fig.I-1). Autocrine products are released into interstitial spaces for actions on the plasma membrane of the cell of origin (Fig. I-1). Neuropeptides with auto- or paracrine functions, transmitting information pre- or postsynaptically in either the central nervous system or in sensory or sympathetic ganglia, operate as neurocrines (e.g. substance P) (Fig. I-1).

According to a new concept, intracrine interaction, a peptide need not be secreted, nor require cell membrane surface receptors, but rather, may remain within the cell of origin and act directly as an intracellular messenger to regulate cell function (e.g. fibroblast growth factor) (Logan, 1990; Lobie *et al.*, 1990; O'Malley, 1990) (Fig I-1).

Diverse Peptide Actions

There are now many examples of the multiple actions of peptides (Kastin *et al.*, 1981; 1987; Said 1981; 1986). In some instances, the central and peripheral actions of the same peptide are complementary, while in others they are opposing (Table I-2). An example of a peptide having different but complementary actions at central and peripheral sites is leuteinizing hormone releasing hormone (LHRH). LHRH stimulates LH and FSH release and has been shown to stimulate mating behaviour in hypophysectomized rats (Moss and McCann, 1973). Subsequently, LHRH has been located centrally in the brain (King and Millar, 1982a; 1982b). In chickens, two translated products of LHRH are present (Mikami *et al.*, 1988); the centrally located product stimulates mating behaviour, the other, LH and FSH release.

An example of a peptide having central effects that oppose its peripheral action is CGRP. Centrally (intracerebroventricularly injected), CGRP causes vasoconstriction and has a vasopressor effect, while peripherally (intravenously injected) it causes vasodilation and has a hypotensive effect.

The actions of a particular peptide need not be related; the same peptide can exert different actions at different sites. For instance, vasoactive intestinal peptide (VIP) acting on the cerebral cortex stimulates glycogenolysis and blood flow, on the adenohypophysis stimulates prolactin secretion, on the adrenal cortex stimulates steroidogenesis, on vascular and bronchial smooth muscle promotes dilation, on intestinal mucosa stimulates chloride ion

secretion and on the pancreatic acini stimulates bicarbonate and amylase release (Said, 1987). Another example is thyrotropin releasing hormone (TRH) which stimulates the release of thyrotropin (TSH) (which, in turn, releases thyroid hormones) and stimulates gastric secretion, suppresses appetite and stimulates respiration (Holtman *et al.*, 1980).

Economy in Diversity

Although the presence in both the nervous and endocrine system of numerous peptides with varied actions operating out of different functional systems, may seem redundant, certain aspects in the generation and functioning of peptides are economical. For instance, the same peptide can exert different actions at different sites (see above), two or more peptides may be generated from the same precursor (*e.g.* pro-opiomelanocortin and VIP/PHI) and alternative splicing of an mRNA yields different peptides (*e.g.* calcitonin and CGRP) Rosenfeld *et al.*, 1983). Furthermore, by their presence in central and peripheral tissues, peptides can efficiently influence or regulate most important body functions including digestion, food intake, drinking, cardiac activity, blood pressure, fluid balance, respiration, stress response, sexual or reproductive functions, blood flow to vital organs and a variety of other metabolic functions (Krieger, 1981; Guilleman, 1985).

THE ONTOGENY AND EVOLUTION OF NEUROPEPTIDES

APUD Hypothesis

The similarities in peptide production and mode of action between nerve and endocrine cells have given insight into their embryological and evolutionary development and diversity. Pearse (1966) noted that a group of four endocrine cells, the pituitary corticotrophs and melanotrophs, pancreatic islet-cells and thyroid parafollicular cells, share a number of cytochemical features, especially the production of biogenic amines, the uptake of the amine precursor 5-hydroxytryptophan, and its decarboxylation to 5-hydroxytryptamine. On the basis of these amine-handling properties, Pearse coined the acronym APUD (for high *a*mine content, amine *p*recursor *u*ptake and *d*ecarboxylation) for this group of cells. He postulated that these related cells, occurring in diverse locations, had the common basic function of peptide hormone secretion and the common embryologic origin from, perhaps, the neural crest.

Le Douarin (1978) established that many cells exhibiting the APUD characteristics are neural crest derivatives, while others are endoderm derivatives. This does not nullify the APUD hypothesis entirely, for a common ancestral origin for endoderm and neural crest cells could be cells from the presumptive ectoblast which are initially programmed for neuroendocrine function. However, others argue that changes in patterns of gene expression during development can account for the presence of the same molecule in both neurons and endocrine cells, since all cells in the body have the capacity to

produce a particular peptide or protein by virtue of identical genomes (Buchanan, 1982).

The Ancestral Neuron

Peptides occur in all uni- and multicellular animals and a particular peptide may exist in more than one animal or phylum. The appearance of peptides throughout the animal phyla may be due to the evolutionary advantage peptides have over other chemical messengers, in that recombination, genetic duplication and mutations of their genes allows for relatively rapid adaptation to the selective forces of a changing environment (Joose, 1987).

One of the most simply organized animals in which neuropeptides have been identified, the polyp *Hydra* (Schaller *et al.*, 1984), has no conventional neurotransmitters or endocrine glands; the nervous system performs all existing endocrine functions. This "ancestral neuron" has the dual entity of long distance and local synaptic chemical signalling. Thus, these nerve-based peptides may be regarded as the phylogenetically oldest blood-bourne messengers in the functional capacity of neurotransmitter and hormone. Despite the subspecializations seen with more advanced species possessing a developed endocrine and nervous system, the close functional association between hormones, neurohormones and neurotransmitters, or between nerve and endocrine cells is fully maintained (Scharer, 1966).

SUMMARY

The name assigned to a peptide usually depicts its originally described function and/or location. The name, therefore, can discourage further investigation of a peptide's function or location. Despite this limitation of nomenclature, an increasing number of peptides have been identified in 'ectopic' locations in which they have 'novel' roles.

PARATHYROID HORMONE

INTRODUCTION

The parathyroid gland was first anatomically identified in the adult Indian rhinoceros in 1852 by a comparative anatomist (reviewed by Maluf, 1980). Later, a correlation between abnormalities of the human parathyroid gland and disease of the bone was drawn. This eventually led to the discovery of the peptide hormone, parathyroid hormone (PTH), responsible for mediating the role of the parathyroid gland in increasing serum calcium levels (Collip, 1925).

Parathyroid hormone has many diverse biological effects. The first and best studied are its physiological actions in regulation of calcium and phosphate homeostasis. However, recent progress suggests that PTH has diverse effects on the functions of bone, lipid, adrenal, muscle and nerve tissues. As well, the receptor-mediated secondary mechanisms that give PTH its actions are being elucidated. However, the lack of purified PTH receptor has somewhat hindered this area of study.

Like many other peptide hormones, PTH is initially synthesized in the form of a larger precursor. Some of the sequences of the genes being transcribed and the mRNA being translated to form this precursor, as well as processing into its final secretory form are known. Although factors causing PTH synthesis and secretion are well understood, the mechanisms involved are not.

ANATOMY OF THE PARATHYROID GLAND

Gross Anatomy

Parathyroid glands are tan to reddish brown encapsulated structures, located on the body of the thyroid gland. In mammals, four parathyroids are usually found, two superior glands on the posterior aspect of the upper thyroid pole, and two inferior glands on the ventral aspect of the lower thyroid pole (Woolfe, 1989). In some mammals, such as rat, the inferior glands may be absent or too small to identify even with the aid of a dissecting microscope. In non-mammals, such as amphibians and avians, usually two glands are symmetrically located posteriorly on either branch of the ascending carotid artery, unassociated with the thyroid gland. No encapsulated parathyroid gland has been identified in any fish species; the parathyroid glands appear in evolution concomitant with the move of vertebrates to a terrestrial, calcium depleted environment.

The Chief Cells

These polyhedral shaped cells predominate the epithelial cells of the parathyroid glands. Individual chief cells functionally pass through synthesis, storage, secretory and resting phases. During

sustained hypocalcemia, the number of cells in active phases of synthesis and secretion increases, evidenced by the reduced size of lipid vacuoles. Immunohistological staining of PTH in the chief cells has not been accomplished. Despite this, ultrastructural characterization, through biosynthetic studies of PTH and inferences taken from other cells, has resulted in the conception of a plausible process by which PTH is synthesized and secreted relative to the organelles within chief cells (Fig. I-2). PTH synthesized and secreted by this proposed process can account for many of its biological actions on peripheral targets.

ACTIONS OF PTH

Calcium and Phosphate Metabolism

The majority of calcium (99 percent) and phosphate (85 percent) in the body exists as insoluble hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$), the principle component responsible for the mechanical properties of bone. However, adequate amounts of soluble calcium and phosphate must be available for normal rates of bone mineralization to avoid bone disease. The soluble fraction of total calcium (less than 1 percent, 1.3 mM) and phosphate (less than 10 percent, 0.05 mM) are crucial for a remarkable variety of other regulatory and metabolic functions (Table I-3), which can be illustrated by the wide range of serious symptoms associated with disorders in their homeostasis (Table I-4).

Homeostatic control of extracellular calcium and phosphate concentrations is accomplished through regulating molecular

mechanisms maintaining intracellular concentrations. A 10,000-fold gradient between intra- and extracellular ionized calcium is driven by a low affinity, high capacity Na^{+}/Ca^{++} exchanger driven by the transmembrane sodium gradient and a high-affinity, low capacity Ca^{++}/H^{+} ATPase (Rasmussen and Barrett, 1984). Analogous, but poorly defined mechanisms maintain the highly compartmentalized intracellular distribution of calcium between the mitochondria, endoplasmic reticulum, calcium sequestering compartments, nucleus and cytosol (Alberts *et al.*, 1990). The entry of calcium into cells may occur via passive diffusion, facilitated diffusion, voltage-dependent channels, Na^{+}/Ca^{++} exchangers and agonist/receptor mediated diffusion (Bringhurst, 1989).

Phosphate intra- and extracellular concentrations are generally comparable (1-2 mM). Movement into the cell may occur through an enzymatic process (Travis and Sugarman, 1971) or through a Na^{+}/phosphate antiporter mechanism, powered by the strong sodium gradient (Quamme *et al.*, 1989a; 1989b; 1989c). Outward phosphate movement may occur through similar mechanisms to those of calcium (Bringhurst, 1989).

By directly or indirectly altering the basal activity of the molecular control mechanisms of intracellular calcium and phosphate homeostasis in specialized cells of the kidney and intestine, PTH has a role in regulating extracellular calcium and phosphate ionic concentrations.

PTH Actions on the Kidney and Intestine

Parathyroid hormone affects the transtubular transport of phosphate and calcium in cells in anatomically discrete regions of the proximal and distal tubules of the kidney nephron (Rosenblatt *et al.*, 1989). PTH exerts a strong phosphaturic action by direct inhibition of Na^+/phosphate antiporter and a strong hypercalcemic action by stimulation of calcium transporters (Rosenblatt *et al.*, 1989). These actions involve protein kinases C and A activated by the products of adenylate cyclase and phospholipase C, following stimulation by hormone-receptor interaction at the cell surface.

The other major renal action of PTH is the stimulation of 25(OH)-vitamin D-1-hydroxylase in specific cells of the proximal tubule. The result is the conversion of 25(OH)vitamin D_3 (low activity) to $1,25(OH)_2$vitamin D_3 (high activity) which is responsible for increased synthesis of cytosolic vitamin D-dependent calcium binding protein (CaBP), in the intestine; increased CaBP correlates extremely well with the increased absorption rate of calcium (Bronner *et al.*, 1986). Active $1,25(OH)_2$vitamin D_3 also increases the number of phosphate carriers (Danisi and Straub, 1980) and the activity of Na^+/K^+ ATPase in the intestinal basolateral membrane, thereby increasing phosphate transport via the Na^+/phosphate antiporter (Cross and Peterlik, 1984).

Although, the effects of PTH on the intestine are largely mediated through $1,25(OH)_2$vitamin D, direct stimulation of calcium uptake in perfused chick duodena has been documented (Nemere and Norman, 1986).

PTH Actions on Bone

Parathyroid hormone transfers insoluble calcium and phosphate from the bone to the blood by the inhibition of bone formation and increased bone resorption. PTH affects different cells in this complex tissue, in a somewhat temporal manner. A rapid (within 1 hour) increase in calcium and phosphate levels is taken from surface pools by cells lining the endosteal surface. Later (within 4 hours), osteoclast activity increases; resorption of fully mineralized bone from the interior increases. Sixteen to 24 hours after PTH stimulation, the number of osteoclasts increases, therefore, a corresponding increase in the rate of calcium mobilization also occurs. Whether a cascade of communications between cells or PTH stimulating each cell individually produces the temporal pattern is not known (Rosenblatt *et al.*, 1989).

Summary

The overall effect of PTH on calcium and phosphate metabolism is the increased absorption and resorption of calcium and phosphate, the reabsorption of calcium and the excretion of phosphate.

Non-Calcium-and Phosphate-Regulating Actions

PTH has additional actions that do not directly influence calcium and phosphate homeostasis. Although it is possible that certain aspects of these "novel" functions (Kobe, 1987) play an integral role in calcium and phosphate metabolic control (Young *et al.*, 1988), it is likely that these actions are newly discovered manifestations of PTH activity.

Novel actions of PTH have been described in the following tissues:

Bone. Bone formation and resorption rates are maintained in balance over a wide range of bone turnover rates and are closely associated at the level of the bone "remodelling unit", suggesting that formation is physiologically coupled to resorption (Linkhart and Mohan, 1989). The number of active differentiated osteoblasts present on the bone surface and osteoblastic cell proliferation, are affected by polypeptide growth factors present in bone (Farley and Baylink, 1982; Linkhart *et al.*, 1986). It has since been demonstrated that PTH stimulates the release of bone derived growth factors, insulin-like growth factor-I (IGF-I) and IGF-II (Linkhart and Mohan, 1989) and potentiates osteoblastic cell proliferation stimulated by IGF-I (Spence *et al.*, 1989). As such, PTH stimulates IGF-I and -II production and release from osteoblasts, and these factors act in an autocrine mechanism that links bone formation with resorption.

Adipose Tissue. Patients with hyperparathyroidism may have significantly raised levels of triglycerides (Werner and Low, 1973). PTH has well defined effects on lipid metabolism including a stimulation of adipose tissue lipolysis *in vitro* and an increase of serum glycerol and free fatty acids *in vivo* (Diueke *et al.*, 1987). Thus the normalization of total serum triglycerides by parathyroidectomy (PTX) in hyperparathyroidal patients, may be explained by the effects of PTH on adipose tissue.

Adrenal gland. Parathyroid hormone has been shown to stimulate corticosterone production *in vivo* (Marotta and Lau, 1970; Marotta, 1971; Williams *et al.*, 1974). This effect had been explained by the similarity between region 15-25 of PTH and region 1-11 of ACTH (Parsons, 1976) or by an indirect action through the increased concentration of plasma calcium. However, recent evidence has demonstrated that avian and bovine PTH stimulate cyclic adenosine monophosphate (cAMP) production and steroid production in avian adrenal cells *in vitro*, comparable to that produced by ACTH (Rosenberg *et al.*, 1987). Bovine PTH (3-34), a known PTH antagonist *in vitro* (see Table I-6), inhibits PTH action but not that of ACTH suggesting that PTH actions on the adrenal gland are through specific receptors distinct from those for ACTH.

Striated Muscle. Parathyroid hormone has direct and indirect effects on the contractility of striated muscle. PTH indirectly promotes the skeletal muscle weakness encountered by patients with hyperparathyroidism which is due to PTH-stimulated elevation of serum calcium levels inducing atrophy of neural afferents (Bertonini, 1987). However, in cardiac muscle, PTH has a direct positive chronotropic effect in dogs (Crass *et al.*, 1982), rats (Kurokawa and Katoh, 1982; Tenner *et al.*, 1983) and other animals (Sham *et al.*, 1981; Jahn *et al.*, 1987). There is some evidence that the inotropic effect is cAMP-independent and may be partially mediated by endogenous myocardial noradrenaline (Katoh *et al.*, 1981).

Vascular Smooth Muscle. Collip and Clark (1925) demonstrated a hypotensive action of PTH after intravenous and subcutancous injection. This hypotensive effect was later confirmed by Handler and Cohn (1952), and shown to be the result of vasodilation (Charbon and Holstaert, 1974; Berthelot and Gairard, 1975) in a number of vascular beds of piscine, amphibian, reptilian and avian species (Pang *et al.*, 1986; Mok *et al.*, 1989) (Table I-5).

The specificity of PTH as a vasodilator was indicated by a number of studies. PTH's vasodilating activity was not altered by changes in extracellular calcium concentration (Mok *et al.*, 1989). The intrinsic hypotensive ability of the PTH molecule can be abolished by H_2O_2 oxidation, which generates the methionine sulfoxide form of the two methionines (Katoh *et al.*, 1981), without affecting the hypercalcemic activity of PTH (Pang *et al.*, 1983). Furthermore, the potency of this action is comparable to known hypotensive peptides (Tenner *et al.*, 1980), and pharmacological evidence indicates this activity is not mediated by adrenergic, histaminergic, cholinergic or endothelium-dependent relaxing factor mechanisms (Mok *et al.*, 1989). Specific PTH receptors have been located on vascular smooth muscle cell membranes (Nickols *et al.*, 1990), and the stimulation of these receptors promotes adenylate cyclase activity (Nickols *et al.*, 1986; 1990).

Other Smooth Muscle. Specific, PTH-induced relaxation has also been reported in uterine (Page *et al.*, 1981; Shew *et al.*, 1984 Shew and Pang, 1984), vas deferens (Zhang *et al.*, 1985),

gastrointestinal (Yang *et al.*, 1985; Mok *et al.*, 1987) and trachial tissue preparations (Yen *et al.*, 1983; Pang *et al.*, 1985; Mok *et al.*, 1989).

Central Nervous System. In cases of acute uremia, markedly elevated PTH levels observed were believed to be a major toxin to the central nervous system (CNS), by means of elevating brain calcium content (Guisado *et al.*, 1975; Cooper *et al.*, 1978). Brain synaptosomes prepared from uremic rats had a significant increase in calcium compared to parathyroidectomized (PTX) uremic rats (Fraser and Sarnacki, 1988). Treatment of PTX uremic rats and normal rats with PTH also caused a significant increase in calcium uptake compared to untreated controls. Furthermore, while *in vitro* administration of bovine PTH-(3-34) had no effect, *in vitro* administration of bovine PTH-(1-34) and -(1-84) significantly increased calcium uptake in normal rat brain synaptosomes, independent of cAMP as a second messenger (Fraser *et al.*, 1988). Thus the *in vivo* and *in vitro* effects of PTH are likely to be mediated by centrally located PTH receptors.

Peripheral Nervous System. Unlike rat brain synaptosomes, neuroblastoma cells show a reduction in calcium uptake in response to PTH *in vitro* (Fang *et al.*, 1990). Receptor-mediated dose-dependent inhibition of the long lasting (L) calcium current was induced by PTH in this cell line which is a good model for the transient (T)- and L-type channels, identified in arterial smooth muscle (Pang *et al.*, 1990).

Summary

PTH has novel actions on a variety of tissues. Some of these actions have been noted for their β-adrenergic like effects (Pang *et al.*, 1986; Mok *et al.*, 1989), suggesting that PTH has neuropeptidergic-like activity. However, this activity has yet to be determined under normal physiological conditions, having only been observed in pathophysiological states and under pharmacological conditions.

MECHANISMS OF ACTION

PTH Receptor Interaction

Like other peptides, PTH interacts with specific receptors on the plasma membrane of target tissue cells. The PTH/receptor interactions have been partially characterized by competitive hormone binding and saturation analysis, using a series of hormone fragments, analogues, agonists and antagonists (Table I-6 and Fig. I-3; for review see Caulfield and Rosenblatt, 1990). The PTH receptor has not been purified or cloned, but some physiochemical properties have been identified. The receptor is approximately 70,000 daltons molecular weight (Draper *et al.*, 1982) and is a glycoprotein (Karpf *et al.*, 1987). However, second messengers other than cAMP mediate PTH actions, (Hrusak *et al.*, 1987; Fraser *et al.*, 1988; Quamme *et al.*, 1989a; 1989b; 1989c; Pang *et al.*, 1990) and indications of possible heterogeneity of the second messengers of PTH receptor (Segre *et al.*, 1979) may predispose the need for PTH receptor subtypes (Karpf *et al.*, 1987; Petersen and Bear, 1986).

The possible presence of distinguishable PTH receptor classes will therefore require careful study by multiple approaches, and structural characterization of molecular clones from different tissues. Once cloned, modification by recombinant DNA techniques (e.g. site-directed mutagenesis, domain swapping and/or partial sequence truncation) and the consequences for hormone-binding and signal transduction, could be analyzed in detail (Caulfield and Rosenblatt, 1990).

Second Messengers

The result of specific cell surface interactions of PTH with its receptor is a cascade of intracellular events that ultimately contribute or cause directly the full spectrum of PTH bioactivity. In most target cells, including smooth muscle (Mok *et al.*, 1989), adrenal tissue (Rosenberg *et al.*, 1987), bone (Rodan and Rodan, 1974) and kidney (Chase and Aurbach, 1967; 1968; Michelakis, 1970), cAMP is the second messenger generated through the stimulatory nucleotide regulatory component of the hormone-sensitive adenylate cyclase system (Robishaw *et al.*, 1986). However, other transducers are involved in relaying PTH information.

Calcium. It might be expected that a lowering of extracellular calcium influx would directly inhibit smooth muscle contraction. Pang and colleagues (1984; 1988) observed a decrease in calcium influx in response to PTH administration to vascular tissues *in vitro*. Recently, the central part of the PTH peptide sequence was demonstrated to increase DNA synthesis, as measured by [^{3}H]

thymidine incorporation, of cultured chondrocytes from day old embryonic chicks (Schluter *et al.*, 1989). This effect was independent of cAMP, but did involve calcium ions in some capacity, since the effect could not be mimicked by forskolin (a cAMP stimulator) and could be blocked by EGTA (a calcium chelator).

Phospholipase C Activity. Phospholipase C activation results in the metabolism of phosphoinositides, producing inositol triphosphate (IP_3), diacylglycerol (DAG), and phosphatidyl inositol 4,5-bisphosphate. The DAG and IP_3 then activate protein kinase C and the release of intracellular calcium, respectively (Quamme *et al.*, 1989a; 1989b; 1989c). This pathway has been shown to be activated in the opposum kidney (OK) cell line, in response to PTH stimulation (Hruska *et al.*, 1987; Civitelli *et al.*, 1988). Phospholipase C activation mediates PTH inhibition of Na^+/phosphate co-transport in OK cells, and may not be involved in calcium transport. Therefore, PTH actions on renal tubular cells may involve two separate receptor-mediated second messenger pathways.

Summary

The interaction of PTH with its receptor(s) can activate many intracellular events. These events are diagramatically summarized in Figure I-4.

PTH: PHYSIOCHEMISTRY

"The active principle in this (ox parathyroid extract) produces its effect by causing the calcium content of the blood serum to be restored within normal limits".

-JB Collip (1925)

Primary Structure

The active principle described by Collip (1925) was the first identification of the peptide hormone, PTH. This initial acid extraction of PTH from the parathyroid gland led to the gradual purification of PTH from human, bovine, porcine and avian glands utilizing a variety of chemical and chromatographic techniques (Aurbach, 1959; Rasmussen *et al.*, 1964; Rasmussen and Craig, 1959; 1962; Aurbach and Potts, 1964; Keutmann *et al.*, 1971; 1974; Cohn and Hamilton, 1976; Pines *et al.*, 1984). In early preparations, isohormonal forms of PTH were suspected due to PTH activity in more than one fraction in the final chromatographic separation (Keutmann *et al.*, 1971). However, eventual sequencing by Edman degradation (Edman and Bigg, 1967), indicated that these bioactive fractions were, in fact, fragmented degradation products of a single form of PTH (Brewer and Ronan, 1970; Niall *et al.*, 1974; Brewer *et al.*, 1972; Sauer *et al.*, 1974; Keutmann *et al.*, 1978). Moreover, the amino acid sequences of purified bovine (Brewer and Roman, 1970), porcine (Sauer *et al.*, 1974), human (Keutmann *et al.*, 1978) and chicken PTH (Pines *et al.*, 1984) are

largely homologous, indicating a degree of evolutionary conservation (Fig. I-5).

Secondary and Tertiary Structure

Secreted PTH is a single-chain polypeptide of 84 amino acids (88 amino acids for chicken) of approximately 9,300 daltons (Rosenblatt *et al.*, 1989). The molecule has a high number of basic residues in the amino acid composition, providing an overall basic nature. There are no cysteine residues for possible disulfide-bridging, but other secondary and tertiary structures have been estimated by alternative methods and predictive formulas (Cohn and MacGregor, 1981).

PTH has not been crystallized, and the three dimensional structure has, therefore, not been ascertained. Based on data from gel migration of terminal fragments (Cohn *et al.*, 1974), high resolution dark-field electron microscopy (Fiskin *et al.*, 1977) and location of α-helix, random coil, β-sheet and β-turn regions by predictive methods (Chou and Fasman 1974a; 1974b; 1977), a three dimensional model was proposed. It consists of the amino-terminal region occupying one domain, the carboxy-region the other, with the two connected by a short stalk which, as observed, would be susceptible to proteolytic action (Fig I-6). Nuclear magnetic resonance (Bundi *et al.*, 1978; Smith *et al.*, 1978) and circular dichroism (Cohn and MacGregor, 1981) suggest that the proposed ordered structure exists when residing within lipid-protein

membranes and when it reacts with membrane-bound receptors, but not in aqueous solution.

PTH Metabolites

Proteolysis of PTH often occurs along the short stalk, dividing the molecule into an amino (1-34) and several carboxyl-terminal fragments (Habener *et al.*, 1971). Fragmentation of PTH-(1-84) occurs intraglandularly (Silverman and Yallow, 1973) and peripherally, primarily in the liver and kidney (Hruska *et al.*, 1981) but also by circulating macrophages (Diment *et al.*, 1989). The processing of PTH in the macrophage endosome is remarkable for peptide digestion, in that cleaved fragments are returned to extracellular medium without delivery to lysosomes, a novel route for processing endocytosed peptides (Diment *et al.*, 1989).

The existence of different fragments of PTH and their different half-lives in the circulation, has complicated efforts to derive clinically useful information from measurements of circulating immunoreactive (IR) PTH. Not all IR fragments are bioactive (Segre, 1983). And as such, it was believed that IR measurement of the amino terminal fragment, PTH-(1-34) by RIA or immunometric assays using affinity purified antisera or monoclonal antibodies would provide an accurate account of bioactive PTH (Segre 1983; 1990). However, the description of carboxy-terminal human PTH fragment (hPTH-(53-84)) stimulation of alkaline phosphatase activity in dexamethasone-treated rat osteosarcoma cells *in vitro*, the first action attributed to

carboxy-terminal PTH (Murray *et al.*, 1989), has clouded the measure of "bioactive" PTH once again.

PTH: BIOSYNTHESIS AND SECRETION

Like other peptides, the paradigm of PTH biosynthesis begins with the transcription of its gene, followed by the translation of the mRNA which produces a larger precursor peptide (prepro-PTH). This precursor is then proteolytically cleaved in the endoplasmic reticulum and again in the Golgi apparatus before the final secretory product is packaged and released into circulation.

PTH Gene

The genomic nucleotide sequences for human, bovine and rat PTH (Vasicek *et al.*, 1983; Weaver *et al.*, 1984, Heinrich *et al.*, 1984) and the location of the human PTH gene on the short arm of chromosome 11, have been determined (Naylor *et al.*, 1983). The overall structures of the three genes are remarkably similar. Each contains two intervening sequences (IVS) that interrupt at the exact same nucleotides (Fig. I-7). The larger IVS A, appears first, five nucleotides prior to the start of the prepro-PTH. The smaller, IVS B, separates exon II which encodes the signal sequence necessary for peptide processing (Gilbert, 1981), from exon III which encodes the remaining hormone sequence and 3′ non-coding region (Fig. I-7). The promoter regions of the human and bovine genes are similar as well; both contain a TATA box and a cAMP recognition element (CRE) sequence (TGACGTCA) at nucleotides 28 and 76, respectively, upstream of exon I (Weaver *et al.*, 1984; Deutsch *et al.*, 1988). The

rat promoter also contains a potential CRE (TGACATCA) at position 42, upstream of exon I, but instead of a TATA box, a Goldberg-Hogness promoter sequence (CAATAAAATA) exists upstream at position 27 (Heinrich *et al.*, 1984; Deutsch *et al.*, 1988). The existence of the CRE in the PTH promoter agrees with cAMP regulation of PTH transcription (Cohn and MacGregor, 1981; Rosenblatt *et al.*, 1989).

PTH mRNA

The cloning of the cDNA for bovine (Kronenberg *et al.*, 1979), human (Hendy *et al.*, 1981) and recently chicken PTH mRNA (Khosla *et al.*, 1989) has provided nucleotide sequences that agree with previously determined primary peptide structures. Although the nucleotide sequences between these species of PTH are largely homologous (at least 70%), the 3' non-coding region of the chicken mRNA is more than three times the size of mammalian mRNA (Khosla *et al.*, 1989). However, the translated prepro-sequence is actually smaller (114 amino acids) and the secreted chicken PTH is larger than mammalian PTH (88 amino acids)(Fig. I-5).

Prepro PTH

A PTH precursor was first recognized by the hypercalcemic activity that resides in more than one chromatographic fraction of parathyroid gland extract (Habener and Kronenberg, 1978). It is now recognized that the primary translated product of PTH mRNA is a 115 (114 for chicken) amino acid precursor, prepro-PTH (Kemper *et al.*, 1974; Kronenberg *et al.*, 1979; Hendy *et al.*, 1981;

Khosla *et al.*, 1988). Like other pre-region sequences from secretory protein precursors, the prepro-region of PTH contains at least one positively charged amino acid near the amino terminal end, an uninterrupted stretch of hydrophobic and nonpolar amino acids and finishes with small amino acids just before and after the third residue, proximal to the pre-sequence cleavage site (Rosenfeld *et al.*, 1989). This type of signal sequence is integral in the initial binding and entry into the endoplasmic reticulum of polyribosomes synthesizing proteins for secretion (Wickner and Lodish, 1985). Thus, mutations in the signal sequence cleavage domain of prepro-PTH alter protein translocation, signal sequence cleavage and membrane binding properties (Wiren *et al.*, 1989).

It is believed that after successful docking of the polyribosome and immediately after the completed prepro-PTH synthesis, the "pre", 23 amino acid, sequence is cleaved off by glycyl-lysyl enzymatic activity located in or near the reticular membrane (Habener and Kronenberg, 1978). Once the prohormone is formed it is transferred to the Golgi where conversion to PTH occurs (Cohn and MacGregor, 1981) by trypsin-like removal of the pro-hexapeptide (Goltzman *et al.*, 1976). Further degradation of PTH occurs in the secretory granules (Silverman and Yallow, 1973).

PTH SECRETION

Calcium

A number of factors influence PTH release from the parathyroid gland. The primary agent is calcium [the powerful stimulation of

phosphate can be explained by the fall in levels of ionized calcium associated with phosphate administration (Sherwood *et al.*, 1968)]. Altered serum calcium ionic levels from the normal set point of 1.3 mM are inversely related to the amount of hormone released *in vivo* and *in vitro* (Sherwood *et al.*, 1968; Mayer *et al.*, 1976). This effect occurs within seconds (Cohn and MacGregor 1981), in a "receptor mediated-like" fashion (Nemeth and Scarpa, 1986). A reverse hemolytic plaque assay, which uses complement-mediated cell lysis to detect antigen release (Smith *et al.*, 1986) was employed to demonstrate that individual parathyroid cells are more sensitive to calcium than cell populations (Fitzpatrick and Leung, 1990) and may be a useful sensitive approach in determining other PTH secretagogues.

Catecholamines

Numerous studies both *in vivo* and *in vitro* have shown that epinephrine can increase PTH secretion (Heath, 1980). It was further demonstrated that the transient rise in PTH, even after prolonged doses of epinephrine, was specifically mediated through β-adrenergic receptors.

Vitamin D Metabolites

The study of the modulatory effects of Vitamin D metabolites on PTH secretion has produced contradictory results from several laboratories (Rosenblatt *et al.*, 1989). However, modest decreases in PTH secretion correlate in time with significant decreases in PTH mRNA levels (Cantley *et al.*, 1985). Thus,

prolonged exposure to $1,25(OH)_2$vitamin D_3 may indirectly affect PTH secretion through a primary effect on PTH biosynthesis.

Mechanisms of Release

The parathyroid cell is remarkable in its capable response to slight changes in extracellular calcium concentration. The intracellular mediators responsible remain uncertain, but the second messengers cAMP, cytosolic free calcium, DAG and IP_3 appear to be involved (Rosenblatt *et al.*, 1989).

PTH-LIKE PEPTIDES

INTRODUCTION

The ectopic distribution of immunoassayable polypeptide hormones in endocrine and nonendocrine tissues has been demonstrated for a number of peptides (see Neuropeptides: The Paraneural System, above). PTH immunoreactivity (IR) has been found in human cerebrospinal fluid (Balabanova *et al.*, 1984), and in tissue homogenates and media containing the brain and pituitary gland of sheep (Balabanova *et al.*, 1985; 1986). Similarly, PTH IR has also been measured in the serum of fish which lack encapsulated parathyroid glands (Harvey *et al.*, 1987). The brain, pituitary and corpuscle of Stannius (CS), an encapsulated gland associated with the teleostean and holeostean kidneys, have been proposed as sources for circulating IR PTH in fish, (Parsons *et al.*, 1978; Milet *et al.*, 1980; 1982; Harvey *et al.*, 1987). Specific antisera have demonstrated the presence of IR PTH in all three tissues (Lopez *et al.*, 1981; 1982; 1984; Harvey *et al.*, 1987) and localized IR

perikarya in the preoptic nuclei of the brain, with axons extending to the neurohypophysis, terminating at the tips of finger-like projections intertwined with pituitary cell bodies (Kaneko and Pang, 1987).

Interestingly, PTH has hypercalcemic effects in mammalian vertebrates (see Action of PTH, above) but, paradoxically, has hypocalcemic effects in piscine vertebrates (Wendelaar-Bonga *et al.*, 1986). Recently, two novel peptides have been isolated based on their PTH-like actions. A hypocalcemic factor, stanniocalcin, has been purified from the CS of salmon (Wagner *et al.*, 1986), eel (Butkus *et al.*, 1987), and trout (Lafeber *et al.*, 1988), while a hypercalcemic factor, PTH-related protein (PTHrp), has been purified from a human lung cancer cell line (Mosely *et al*, 1987; Suva *et al.*, 1987) and malignant and normal rat tissue (Yasuda *et al.*, 1989b). The existence of these PTH-like peptides raises the possibility that the IR PTH measured may be more like stanniocalcin or PTHrp rather than PTH.

STANNIOCALCIN

Corpuscles of Stannius

The corpuscles of Stannius (CS), small encapsulated whitish glands, were mistakenly assumed to be homologues of mammalian adrenal glands because of their close association with the kidneys of holostean and teleostean species (Stannius, 1839). It was later demonstrated that these secretory granule-containing glands bore no relation, either embryologically or morphologically, to mammalian

adrenals (Wendelaar-Bonga and Pang, 1982). Furthermore, the CS are present only in species without parathyroids. It is now known that CS ablation causes plasma hypercalcemia in high-calcium environments and that at least one factor produced by the CS inhibits branchial calcium ion transport (Fontaine, 1964; 1967; Chester-Jones *et al.*, 1965; Pang, 1971; 1973; Pang and Pang, 1974; Pang *et al.*, 1973; 1974; 1975; 1980; Fenwick, 1974; 1976; Fenwick and So, 1974; So and Fenwick, 1977; 1979).

Teleocalcin, Stannius Protein, Hypocalcin and Parathyrin of the CS

Several candidates for hypocalcemia-causing factors have been proposed. Ma and Copp (1978) originally proposed that the factor was a glycoprotein of 3 KDa, while Fenwick (1982) determined that the minimum size was 10 KDa. Some evidence suggests that this factor is related to PTH on the basis of immunological (Milet *et al.*, 1980; 1982; Lopez *et al.*, 1981; 1982; 1984; Harvey *et al.*, 1987) and bioactivity studies (Lafeber *et al.*, 1986; Wendelaar-Bonga *et al.*, 1986). Five candidates responsible for hypocalcemic activity in response to hypercalcemia have been identified.

"Teleocalcin": Two glycoprotein hormones of the same molecular weight (32 KDa) and which are disulfide linked oligomers, were purified from sockeye (*Oncorhynchus nerka*) (Wagner *et al.*, 1986) and coho (*O. kisutch*) salmon corpuscles of Stannius (Wagner *et al.*, 1988). The two hormones are similar on the basis of amino acid and carbohydrate composition and 95 percent sequence homology in the first 40 amino-terminal residues, but they do not

co-elute from a concanavalin A-Sepharose column. Both teleocalcins had potent inhibitory effects on gill calcium uptake in intact rainbow trout at the peak in the seasonal uptake of calcium cycle. In stanniectomized (STX) eels, either teleocalcin acutely reduced or abolished the post-operative accelerated calcium transport. No immunoreactivity with PTH antisera or amino acid sequence homology was indicated.

"Stannius protein": A major glycoprotein of 32 KDa was purified by electrophoresis and electroelution, from the eel (*Anguilla australis*) CS (Butkus *et al.*, 1987). A 75-mer oligonucleotide probe was designed from the partial amino-terminal sequence and used to screen a CS cDNA library (Butkus *et al.*, 1987). The cDNA coded for a 231 amino acid, 24,632 Da glycoprotein with a sequence 80% homologous with coho and sockeye teleocalcin in the first 40 amino-terminal residues (Wagner *et al.*, 1988). There was no sequence homology with PTH or other proteins, including renin or osteocalcin (Butkus *et al.*, 1987).

"Hypocalcin": An hypocalcemic-causing glycoprotein of 54 KDa was purified from the CS of trout (*Salmo gairdneri*) (Lafeber *et al.*, 1988). When electrophoresed on a reducing gel (β-mercaptoethanol), the 54 KDa protein appeared as a single 28 KDa band. This suggests that the 54 KDa protein is a 28 KDa dimer held together by disulfide bridging. Furthermore, no immunoreactivity with PTH antisera or primary structure similarities with PTH were measured, but the first 33 amino acid residues matched perfectly with those of *Coho* teleocalcin.

"Parathyrin from the CS (PCS)": Partial purification of PCS by reverse phase HPLC and PTH-affinity chromatography, showed that a biologically active (alkaline phosphatase stimulation in eel gut homogenate) peak eluted in 32% acetonitrile (similar to hPTH-(1-84)) and contains a 32 to 34 KDa protein. When given to STX eels, the purified fraction was 600-fold more hypocalcemic than the crude CS extract (Milet *et al.*, 1989). The total RNA extracted from eel CS also produced a 45 KDa protein, immunoprecipitable by PTH antisera, believed to be a precursor for the 32 KDa protein. Milet *et al.* (1989) proposed that this 32 KDa PCS may be a differentially processed ancestral PTH gene product from that observed for mammalian and avian PTH.

Summary

The close homology in sequence and bioactivity of the salmon, eel and trout hypocalcemic factors, suggest that they are species-specific variations of a common 54 KDa dimer glycoprotein, stanniocalcin. The stanniocalcin molecule is unlike PTH immunologically and structurally. However, the hypocalcemic-causing factor partially purified by Milet *et al.*, (1989) resembles PTH structurally (elution profile) and immunologically (affinity chromatography and immunoprecipitation). The relationship of PCS with stanniocalcin is not known, and awaits PCS sequencing.

PTH-RELATED PEPTIDE

The existence of a PTH-like factor mediating hypercalcemia associated with malignancy was postulated based on the coordinated

occurrence of hypophosphatemia with hypercalcemia in a cancer patient (Goltzman *et al.*, 1989). Subsequent studies attempting to identify the presence of PTH immunochemically (Powell *et al.*, 1973) or by Northern blot analysis (Simpson *et al.*, 1983) failed. However, *in vivo* and *in vitro* bioassays for PTH confirmed the presence of a PTH-like substance circulating within patients with malignancy-associated hypercalcemia (Stewart *et al.*, 1980; Goltzman *et al.*, 1981). These assays were used as indices for the protein purification and partial sequencing of peptides from a human lung cancer cell line (Moseley *et al.*, 1987), human breast cancer (Stewart *et al.*, 1987) and a human kidney cancer cell line (Strewler *et al.*, 1987). The amino acid sequence homology of the isolated peptides confirmed the existence of PTH-related peptide (PTHrp). The sequences were used to synthesize oligonucleotides for the molecular cloning of cDNA's encoding PTHrp, from which the primary structure was predicted (Suva *et al.*, 1987).

PTHrp Structure and Biosynthesis

The human cDNA's predict the structure of three isoforms of the mature human peptide, having 139, 141 and 173 amino acids. Analysis of the PTHrp gene, has demonstrated how the mRNA's may be derived from a single gene by alternative splicing (Mangin *et al.*, 1989; Yasuda *et al.*, 1989). The amino acid sequence of the mature forms of PTHrp displays a high degree of homology with the amino-terminal 13 residues of PTH. The first four amino acids of

PTHrp and bovine and rat PTH are identical; within this region hPTHrp differs from the human, porcine and chicken PTH molecules only in the substitution of alanine for serine at position 1. Overall, 7 to 9 of the first 13 residues of PTHrp are identical with those of PTH, depending on the species. Furthermore, the prohormone cleavage site (Lys-Arg) is retained in all known species of PTHrp and PTH. The remainder of the PTHrp sequences, including prepro-regions, show very little homology with those of PTH (Fig. I-8).

Actions of PTHrp

In view of the discovery of PTHrp as a factor emulating PTH effects, and its sequence homology with PTH, evaluation of PTHrp bioactivity has focused on its PTH-like actions. Although modest discrepancies have been noted between PTHrp and PTH actions in various *in vitro* renal or osseous systems, measuring receptor-binding and post-receptor actions, overall, the two peptides appear very close in potency efficacy and range of activities (Goltzman *et al.*, 1989). Despite the absence of significant amino acid sequence homology beyond residue 13, tertiary structures of the amino terminal, modelled after NMR studies, suggest overall structural features in common with PTH (Barden and Kemp, 1989) and may explain the remarkable similarities in actions of PTHrp and PTH.

PTHrp Distribution

In addition to tumorous tissue, PTHrp has now been located in normal tissues. The presence of PTHrp in fetal tissues (Rodda *et al.*, 1988), lactating mammary glands (Thiede *et al.*, 1988;

Thiede and Rodan, 1988) and normal adult tissues, such as keratinocytes (Ikeda *et al.*, 1988), gastric cells (Yasudo *et al.*,) and brain (Weir *et al.*, 1990), may faciliate normal physiological roles for PTHrp in modulating calcium homeostasis or certain brain activity.

Summary

Although the exact physiological role of the PTHrp has yet to be defined in any tissue, the role may be predominantly local (paracrine and/or autocrine) rather than systemic. It may be that the only circumstance in which PTHrp enters the systemic circulation in sufficient quantity to exert a conventional endocrine effect is in the specific pathological setting of humoral hypercalcemia of malignancy (Weir *et al.*, 1990).

OBJECTIVE

Preliminary immunoreactive studies have shown that PTH may be present in fish and mammalian brain. It is the purpose of this thesis to,

1. Identify and measure PTH and PTH-related peptides in central and peripheral nervous tissue,
2. Separate PTH and PTH related peptides from other components of the brain and pituitary,
3. And determine the expression of the PTH and PTH related genes in central nervous tissue.

Table I-1 Mammalian brain peptides

Hypothalamic-releasing hormones
- Thyrotropin releasing hormone
- Gonadotropin-releasing hormone
- Somatostatin
- Corticotropin-releasing hormone
- Growth hormone-releasing hormone

Neurohypophyseal hormones
- Vasopressin
- Oxytocin

Pituitary peptides
- Adrenocorticotropic hormone
- β-Endorphin
- α-Melanocyte-stimulating hormone
- Prolactin
- Luteinizing hormone
- Growth hormone
- Thyrotropin

Opioid peptides
- Dynorphin
- β-Endorphin
- Met-enkephalin
- Leu-enkephalin

Invertebrate peptides
- FMRF amide
- Hydra head activator

Gastrointestinal peptides
- Vasoactive intestinal peptide
- Cholecystokinin
- Gastrin
- Substance P
- Neurotensin
- Met-enkephalin
- Leu-enkephalin
- Insulin
- Glucagon
- Bombesin
- Neurophysins
- Secretin
- Somatostatin
- Thyrotropin
- Motilin
- Pancreatic polypeptide
- Calcitonin
- Calcitonin gene-related peptide
- Gastrin releasing peptide

Growth factors
- IGF-I
- IGF-II
- EGF
- FGF
- NGF
- Endothelial cell growth factor

Others
- Angiotensin-II
- Bradykinin
- Carnosine
- Activin
- Inhibin
- Neuropeptide Y
- PHI
- Atrial natriuretic peptides
- Tachykinins
- Xenopsin

References: Krieger (1984) and Said (1987)

Table I-2 Examples of complementary and opposing peptide central and peripheral actions

Peptide	Central effect	Peripheral effect
Complementary actions		
ANP	Acts on brain water and electrolyte regulatory centres to reinforce influence on blood pressure and fluid balance	Reduces peripheral vasculature tone and promotes glomerular filtration and natriuresis (Said, 1986)
Angiotensin-II	Acts on subfornical organ neurons	Causes vasoconstriction on vascular smooth muscle (Said 1986)
CCK and Bombesin	Suppresses hunger and satiety	Suppresses hunger and satiety (Morley *et al.*, 1984; 1985)
CRF	Stimulates secretion of ACTH and glucocorticoids	Stimulates secretion of noradrenaline from the adrenal medulla and sympathetic ganglia (Brown *et al.*, 1985)
GH	Increases feeding response	Causes numerous direct and indirect effects with growth promotion (Said 1987)
IGF's	Promotes mitogenic growth	Promotes mitogenic growth (Said, 1986)
LHRH	Stimulates mating behavior	Stimulates the release of FSH and LH from the adenohypophysis (Moss and McCann, 1973; Kastin *et al.*, 1987)

Table I-2 Cont'd

<u>Opposing effects</u>		
Bradykinin, CGRP and Substance P	Promotes a vasoconstrictive, vasopressive effect	Promotes a vasodilative, hypotensive effect (Said, 1986)
Bombesin and GRP	Inhibits meal-stimulated gastric secretion	Stimulates gastrin release and acid secretion (Morley *et al.*, 1984; 1985)
Enkephalin	Promotes analgesia	Does not promote analgesia (Hughes *et al.*, 1975; Kastin *et al.*, 1976)

Table I-3 Physiological roles of calcium and phosphate

Calcium	Phosphate
Extracellular	Constituent of biological molecules
1. [illegible] formation	1. Phopholipids (phosphoinositides)
2. Coagulation of blood	2. Phosphoproteins (kinases)
3. Neuromuscular functions	3. Nucleic acids
	4. Enzyme co-factors (NADP)
	5. Glycolytic intermediates
Intracellular	Functions of these molecules
1. Second messenger for extracellular regulators	1. Structural (phospholipids)
2. Coordinator of cellular metabolic activity (effects on calmodulin activity)	2. Energy metabolism and storage (ATP)
	3. Growth
	4. Information transfer (cAMP)
	5. Ion and Protein transport (ATPase)
	6. Muscle contraction
	7. Nerve impluse transmission (Na^+/K^+ ATPase)

Reference: Bringhurst, 1989

Table I-4 Symptoms associated with calcium and phosphate disorders

Hypocalcemia	Hypophosphatemia
Tetany	Muscle weakness
Distinct EEG pattern changes (may correlate with convulsive seizures)	Neurological disorders (confusion to coma to death)
Skin and hair disorders	Renal tubular dysfunction
Cataracts	Hemolysis
Prolonged systole and possible congestive cardiac failure	Poor bone formation
Poor bone formation	Acidosis
(Parfitt, 1989)	(Bringhurst, 1989)
Hypercalcemia	Hyperphosphatemia
Polyuria	Parasthesias
Polydypsia	Muscle cramping
Dehydration	Tetany
Renal compromise	Mental disorders
Neurological dysfunction (from lethargy or confusion to coma)	Prolonged systole
	Renal compromise
(Segre and Potts, 1989)	(Bringhurst, 1989)

Table I-5 PTH as a vascular smooth muscle relaxant

Preparation	Reference
Rat tail artery	Pang *et al.*, 1986
Rat aortic strips	Nickols *et al.*, 1986
Rat mesenteric vessels	Nickols *et al.*, 1986
Rabbit aorta	Nickols and Cline, 1987
Rabbit renal artery	Caulfield *et al.*, 1988
Bovine cerebral artery	Suzuki *et al.*, 1983
Human cerebral artery	"
Bovine basilar artery	"
Porcine basilar artery	"
Chicken mesenteric artery	Pang *et al.*, 1984
Rooster *(in vivo)*	Pang *et al.*, 1980
Bullfrog *(in vivo)*	"
Lungfish *(in vivo)*	"
Snake *(in vivo)*	"

Table I-6 Examples of known agonists and antagonists of PTH bioactivity *in vitro**.

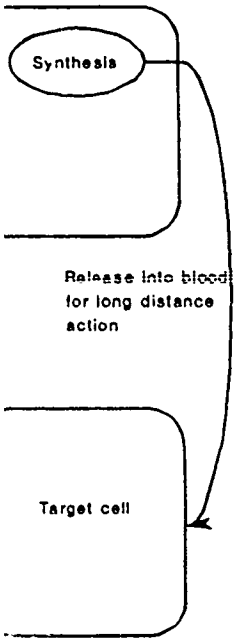
Agonists	Relative potency (%)[a]
bPTH-(1-84)	100
bPTH-(1-34)	100
[Tyr^{34}]-bPTH-(1-34)	139
[$Nle^{8,18}$,Tyr^{34}]-bPTH-(1-34)	76
[$Nle^{8,18,125}$1-Tyr^{34}]-bPTH-(1-34)	69
Antagonists	**K_i(nM)[b]**
bPTH-(3-34)*	5300
[$Nle^{8,18}$,Tyr^{34}]-bPTH-(3-34)*	160
[$Nle^{8,18}$,o-NPS Tyr^{23}, Tyr^{34}]-bPTH-(3-34)*	90
[$Nle^{3,18}$bPTH-(7-34)-NH_2	1550
[$Nle^{8,18}$,Leu^{23},Tyr^{34}]-bPTH-(7-34)-NH_2	>7100
[D-Trp^{12},Tyr^{34}]-bPTH-(7-34)-NH_2	70
[$Nle^{8,18}$, D-β-Nal^{12}, Tyr^{34}]-bPTH-(7-34)-NH_2	140

a Relative potency - calculated on the basis of mean potency estimates with activity of reference compound, bPTH-(1-84) (Rosenblatt *et al.*, 1977).

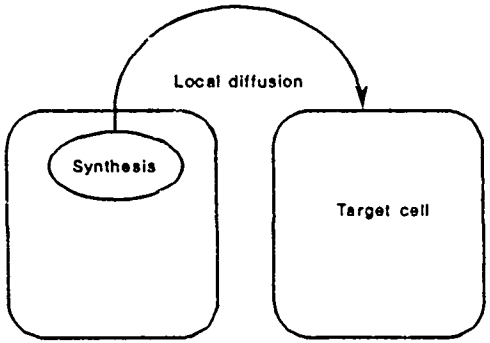
b K_i - inhibitory constant obtained from the dose at which 50% inhibition of native bPTH-(1-84) action occurred (Caulfield and Rosenblatt, 1990)

* These studies were conducted *in vitro*. Studies done *in vivo* suggest that some of the antagonists have partial agonistic activities. These are designated by * (Segre *et al.*, 1985).

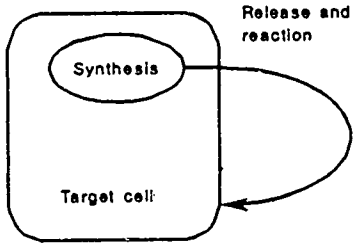
Figure I-1 Modes of peptide regulation of target cells. Endocrine peptides are released into the circulation for actions on distant target cells. Paracrine peptides are released into the interstitial space for actions on local, neighboring cells. Autocrine peptides are released into the extracellular fluid for actions on the cell of origin. Intracrine peptides are not released and have actions on the cell of origin. Neuroendocrine peptides are endocrine peptides originating from neurons. Neurocrine peptides may be auto- or paracrine peptides originating from neurons (Logan, 1989; O'Malley, 1990; Krieger, 1984).



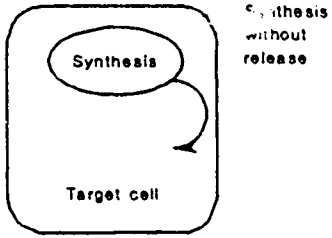
ıdocrine and
ɪuroendocrine



Paracrine and Neurocrine



Autocrine
and Neurocrine



Intracrine

Figure I-2 A schematic representation of a parathyroid chief cell at the ultrastructural level, synthesizing and secreting parathyroid hormone (PTH). 1. The process begins in the nucleus with the promotion of RNA Polymerase II (Pol II) transcriptional activity by cAMP response element (CRE) binding protein (CREB) facilitating TFIID TATA box binding factor to bind to the TATA box. 2. This results in the synthesis of PTH hnRNA which is processed into mRNA (by removal of IVS sequences) and translocated into the cytoplasm. 3. PTH mRNA translation by ribosomal complexes, produces the amino terminal preproPTH signal sequence necessary for endoplasmic association. 4. Once the preproPTH is completely synthesized the prepro-region is enzymatically cleaved to yield proPTH. 5. Subsequently, the pro-region is enzymatically cleaved in the Golgi and packaged into a secretory granule (6.) for exocytosis (7.) into the general circulation where PTH is located for peripheral actions and digestion (adapted from Wolfe, 1989).

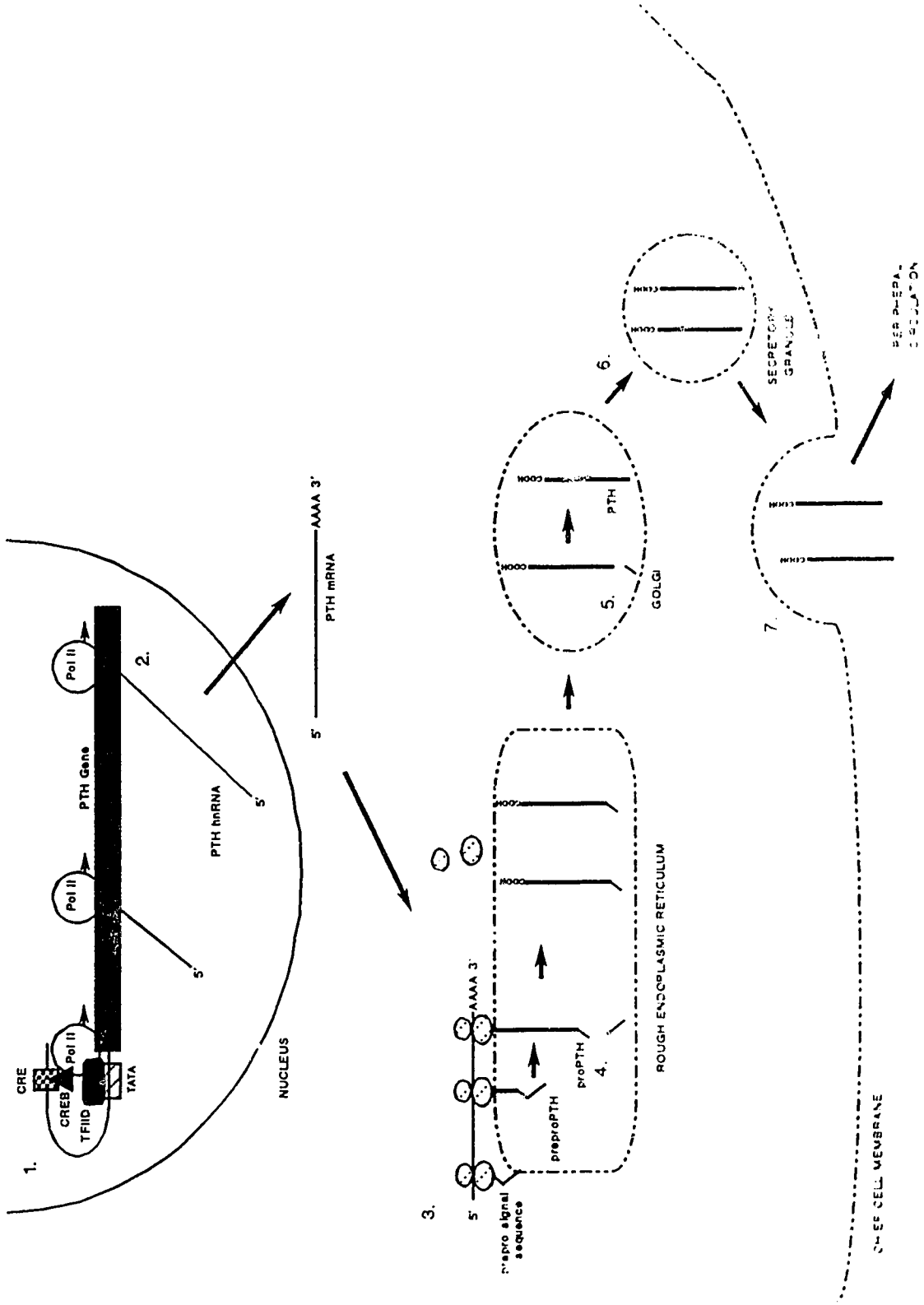

1.
CRE
CREB
TFIID
Pol II
TATA
2.
PTH Gene
PTH hnRNA
5'
NUCLEUS
PTH mRNA
AAAA 3'
3.
AAAA 3'
preproPTH
proPTH
4.
ROUGH ENDOPLASMIC RETICULUM
5.
GOLGI
PTH
6.
SECRETORY
7.
CHIEF CELL MEMBRANE

Figure I-3 A functional map of bovine parathyroid hormone (1-84). The region with full biological activity (1-34) is indicated by the broken boundary. This region may be separated into two domains; an inhibitory domain (7 to 34, shown with bold boundary) that can bind to PTH receptors without activating adenylate cyclase activity *in vivo*, and an activation domain (1 to 7, shown surrounded by the broken boundary) essential for biological activity once binding to the receptor has occurred. Boxed residues depict the 25 to 34 amino acid residues required for receptor occupancy, the principal binding domain (adapted from Rosenblatt *et al.*, 1989).

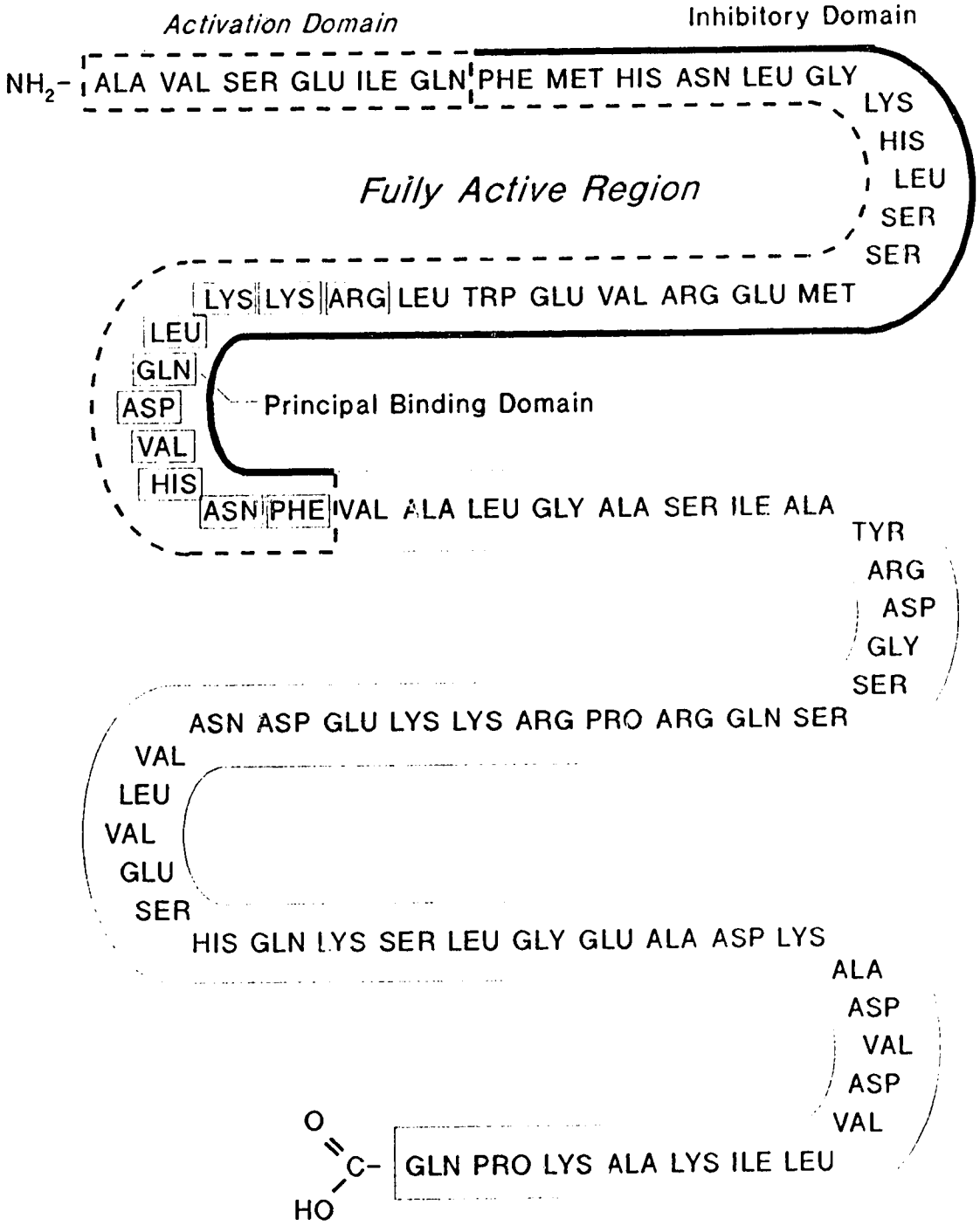

Activation Domain
Inhibitory Domain
NH2- ALA VAL SER GLU ILE GLN PHE MET HIS ASN LEU GLY
LYS
HIS
LEU
SER
SER
Fully Active Region
LYS LYS ARG LEU TRP GLU VAL ARG GLU MET
LEU
GLN
ASP
VAL
HIS
Principal Binding Domain
ASN PHE VAL ALA LEU GLY ALA SER ILE ALA
TYR
ARG
ASP
GLY
SER
ASN ASP GLU LYS LYS ARG PRO ARG GLN SER
VAL
LEU
VAL
GLU
SER
HIS GLN LYS SER LEU GLY GLU ALA ASP LYS
ALA
ASP
VAL
ASP
VAL
O
C-
HO
GLN PRO LYS ALA LYS ILE LEU

Figure I-4 A stylistic summary of PTH second messenger systems. 1. The binding of PTH to its receptor stimulates the conversion of GTP to GDP, which activates the G-protein complex (GS) which in turn stimulates adenylate cyclase activity (AC). The synthesis of cAMP activates protein kinase A (PK-A) which may directly or indirectly phosphorylate (P) cAMP response element (CRE) binding protein (CREB). Phosphorylated CREB then enhances transcription of a gene by binding to CRE. 2. PTH/receptor interaction activates through the Gp protein complex which activates phospholipase C which breaks down phosphoinositide into inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases (↑) intracellular calcium concentration ($[Ca^{2+}]_i$) by stimulating Ca^{2+} release from intracellular stores. DAG activates protein kinase C (PK-C) which inactivates Na^+/phosphate antiporter. 3. Binding of PTH to receptor results in the shutting of ligand-gated calcium channels, thus reducing the influx of extracellular calcium into the cell. More than one receptor type may be necessary (see text).

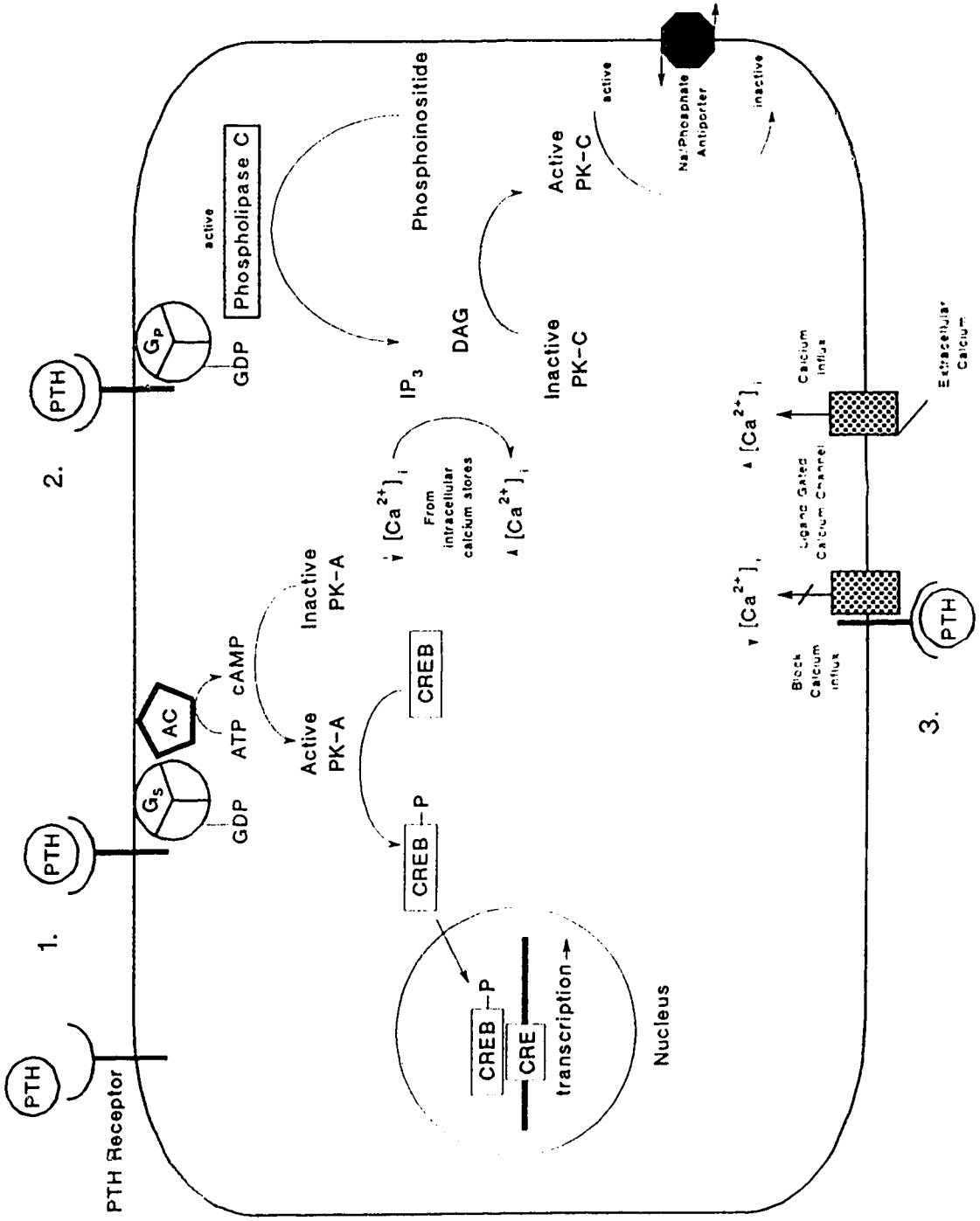
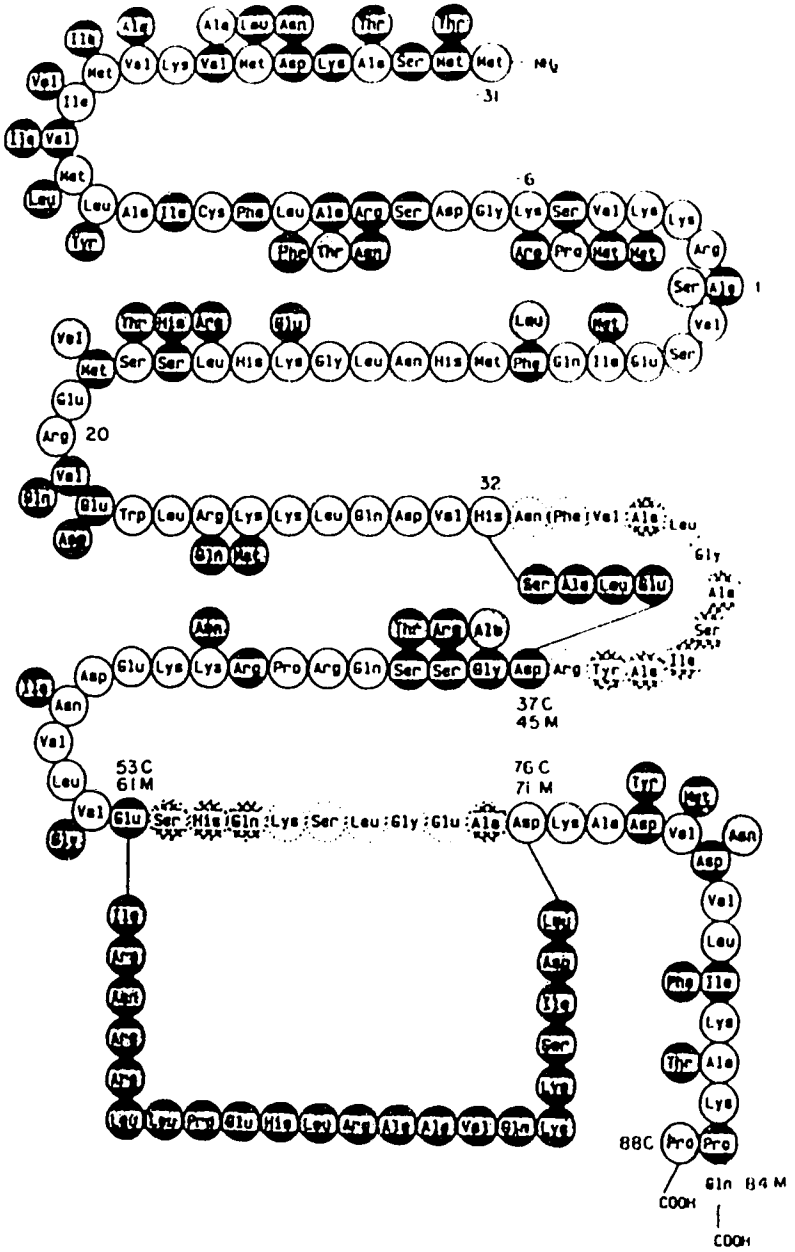

PTH Receptor
PTH
1.
PTH
2.
PTH
3.
G_s
G_p
GDP
GDP
AC
ATP
cAMP
Inactive PK-A
Active PK-A
CREB
CREB—P
CREB—P
CRE
transcription
Nucleus
active
Phospholipase C
Phosphoinositide
IP_3
DAG
Inactive PK-C
Active PK-C
active
inactive
Na⁺/Phosphate Antiporter
$\downarrow [Ca^{2+}]_i$
From intracellular calcium stores
$\uparrow [Ca^{2+}]_i$
$\uparrow [Ca^{2+}]_i$
Calcium influx
Extracellular Calcium
Ligand Gated Calcium Channel
$\downarrow [Ca^{2+}]_i$
Block Calcium influx

Figure I-5 Comparison of predicted chicken prepro-PTH amino acid sequence with those of the mammalian preproparathyroid hormones. The sequence of bovine prepro-PTH is shown in circles. A barred or hatched circle indicates a position at which the amino acid varies among the mammalian hormones (human, bovine, porcine and rat); open circles indicate invariant mammalian residues. Stippled circles indicate sites where the sequence of chicken preproPTH is different from bovine but identical to one of the other mammalian hormones. Barred stippled circles indicate an amino acid unique to chicken preproPTH. Dotted circles represent amino acids that have apparently been deleted in the chicken sequence and replaced by unique peptides, which are joined to the rest of the sequence by lines. The first residue of mature PTH; M and C after numbers refer to the mammalian and chicken sequences, respectively (taken from Khosla *et al.*, 1988).



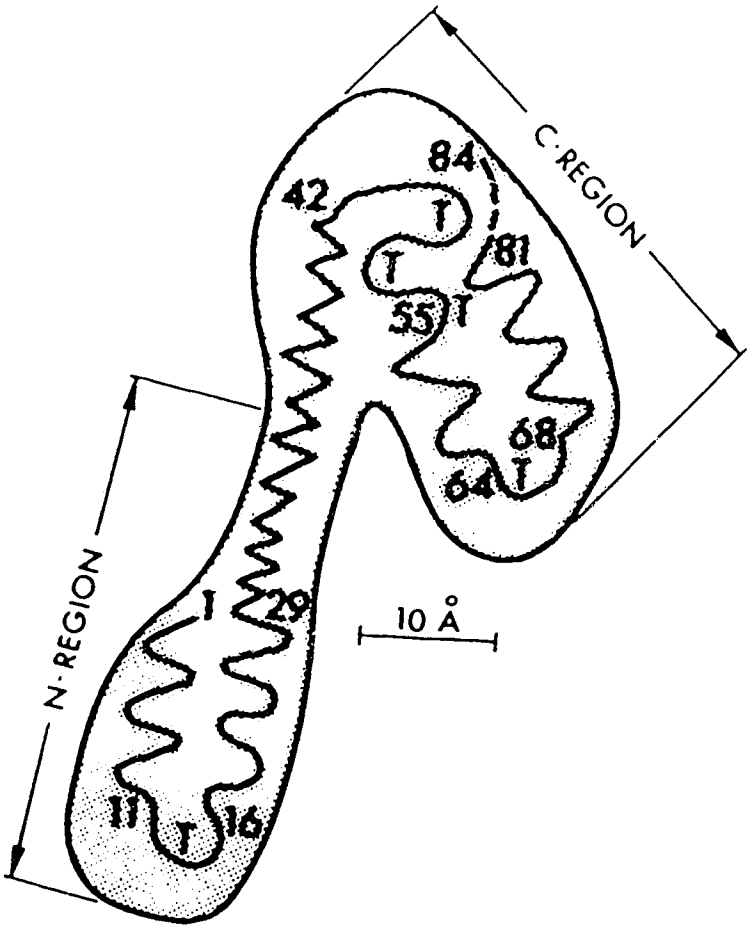


Figure I-6 The proposed tertiary and secondary structure of bovine PTH. The predicted distribution of α-helix (~), β-sheet (⋀⋀), β-turn (⌣) and random coil (---) in bovine PTH according to Fiskin *et al.* (1977).

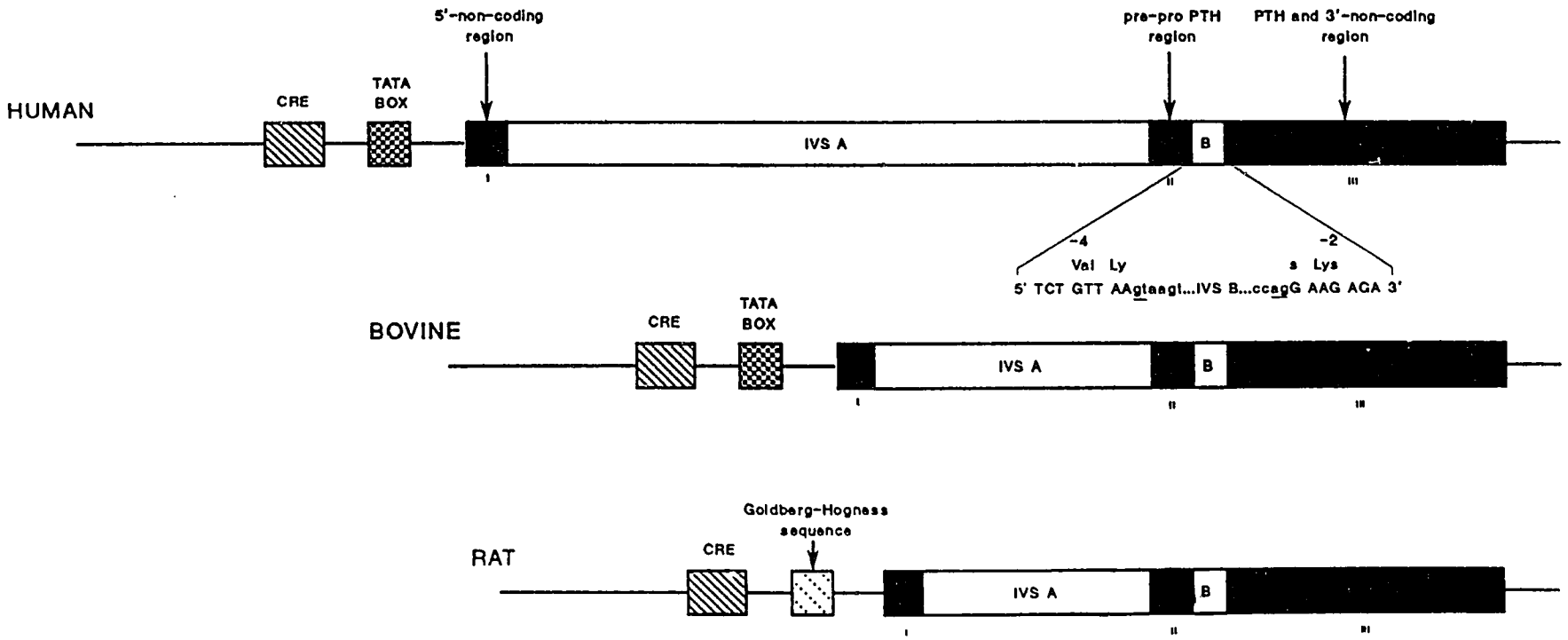


Figure I-7 PTH gene structures. Sequences found in mature mRNA (exons I, II and III) are in black; intervening DNA sequences (IVS A and B) are in white; potential cAMP response elements (CRE) are in diagonal lines; TATA box, TATA binding protein regions, are checkered; and the Goldberg-Hogness sequence is in broken diagonal lines. Magnification of the IVS-B flanking consensus sequences are indicated for human PTH gene.

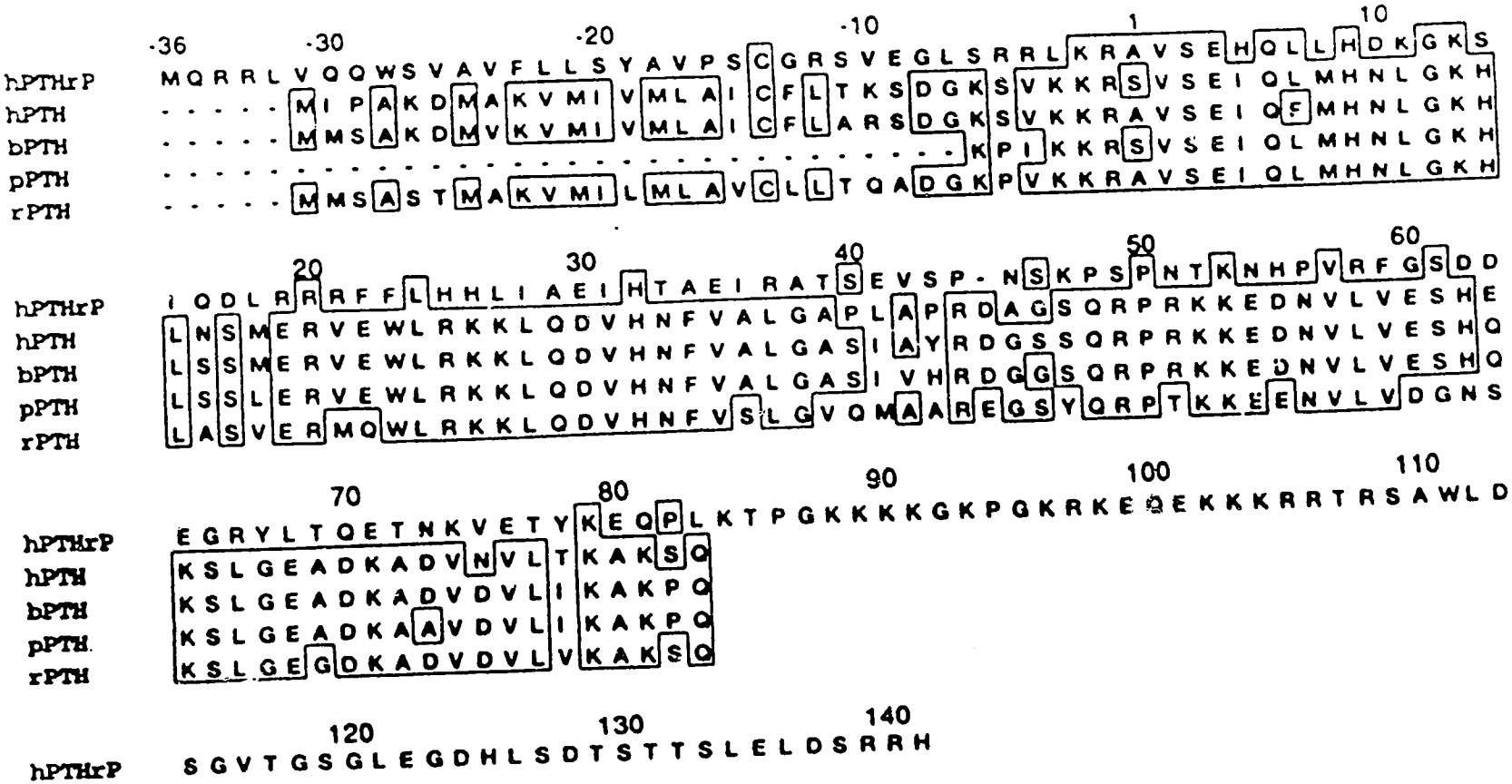


Figure I-8 Homology of PTH-related protein and human (h) bovine (b), porcine (p) and rat (r) PTH (taken from Suva et al., 1987).

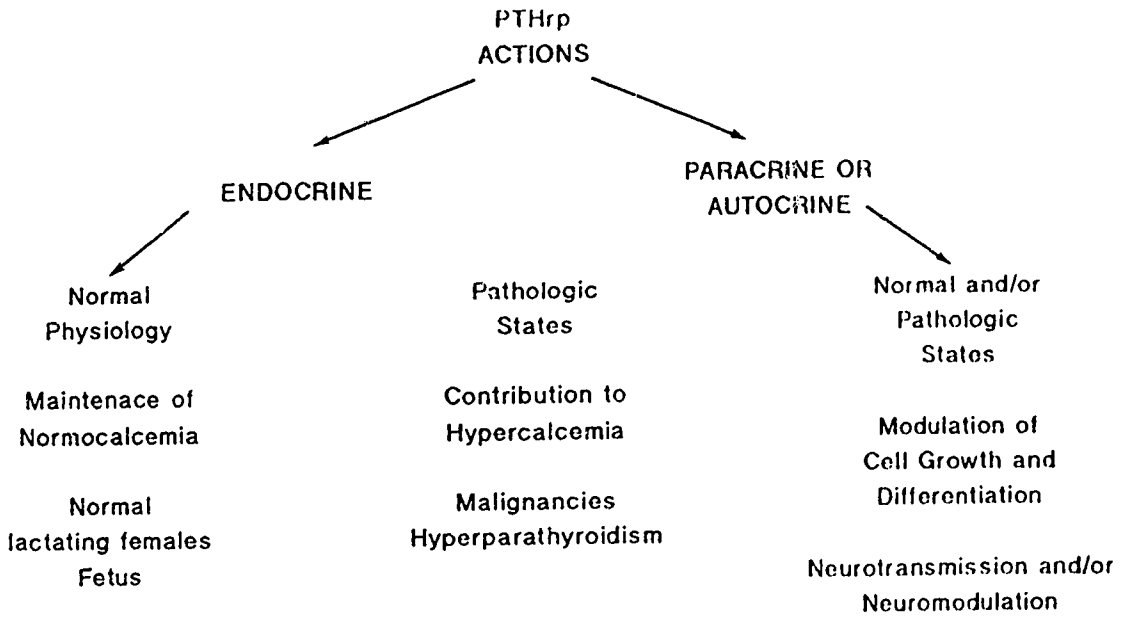


Figure I-9 Potential actions of PTHrp as an endocrine or paracrine/autocrine factor. The best documented action, to date, is as a mediator of the hypercalcemia of malignancy. However, preliminary evidence exists to support a possible role for PTHrp in each of the other functions listed (adapted from Goltzman *et al.*, 1989).

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C H A P T E R I I

CHARACTERIZATION OF ANTISERA RAISED AGAINST STANNIOCALCIN PURIFIED FROM CORPUSCLES OF STANNIUS OF RAINBOW TROUT, *SALMO GAIRDNERI*[1]

1. A version of this chapter has been published. Kaneko T, Fraser RA, Labedz T, Harvey S, Lafeber FPJG, Pang PKT, 1988 Characterization of Antisera Raised against Hypocalcin (Teleocalcin) purified from Corpuscles of Stannius of Rainbow Trout, *Salmo gairdneri*. Gen Comp Endocrinol **69**: 238-245.

INTRODUCTION

The corpuscles of Stannius (CS) are small endocrine glands associated with the kidneys of holostean and teleostean fish. Since Fontaine (1964) found that the removal of the CS induced hypercalcemia in European eel, *Anguilla anguilla*, the CS have been shown to contain hypocalcemic factor(s) (for reviews see Pang *et al.*, 1980; Sokabe, 1982). Pang *et al.* (1974) and Pang and Pang (1974) showed the presence of hypocalcemic activity in the CS of killifish, *Fundulus heteroclitus*, *cod*, *Gadus morhua* and channel catfish, *Ictalurus punctatus*, and named this hypocalcemic principle from the CS "hypocalcin," which has since been renamed stanniocalcin.

Although several candidates for stanniocalcin have been proposed, the precise nature still remains unclear. Ma and Copp (1978) isolated a 3 kDa glycopeptide from the CS of Pacific salmon, which was hypocalcemic in American eel, *Anguilla rostrata*, and named it "teleocalcin." Angiotensin-like substances generated by incubating CS with homologous plasma have also been shown to be hypocalcemic in carp, *Cyprinus carpio*, Japanese goosefish, *Lophius litulon* (Pang *et al.*, 1981), and Japanese eel, *Anguilla japonica* (Ogawa and Sokabe, 1982). So and Fenwick (1982) reported that the CS of American eel contain an acid-stable hypocalcemic factor, and Fenwick (1982) described the primary antihypercalcemic factor of the CS as a protein with a molecular weight greater than 10,000. Furthermore, there is some evidence suggesting that the hypocalcemic factor of the CS is related to parathyroid hormone (PTH) on the basis

of immunological studies: Milet *et al.* (1980, 1982) reported that the hypocalcemic activity of the European eel CS is associated with a molecule resembling mammalian PTH and named it "parathyrin of the corpuscles of Stannius (PCS)."

The CS have been shown to react immunocytochemically with mammalian PTH antibodies (Lopez *et al.*, 1981, 1982, 1984a, b). The immunological similarity between the hypocalcemic factor of the CS and PTH was further confirmed by Harvey *et al.* (1987), who also detected PTH immunoreactivity in the CS of some teleosts. However, Wagner *et al.* (1986) purified a glycoprotein from the CS of sockeye salmon, *Oncorhynchus nerka*, which they called teleocalcin; this molecule does not resemble PTH with respect to amino acid composition and does not exhibit cross-reactivity in their PTH radioimmunoassay.

Recently, Lafeber *et al.* (1987) purified a hypocalcemic substance (stanniocalcin) from the CS of rainbow trout, *Salmo gairdneri*, which was glycoprotein in nature. This isolated glycoprotein has an apparent mass of 54 kDa. In the present study, we raised antisera against this 54-kDa product and characterized them for future use in immunological and physiological studies.

MATERIAL AND METHODS

Immunization Procedure

Antisera were raised against stanniocalcin, a 54-kDa product purified from the rainbow trout CS (Lafeber *et al.*, 1987) in a

male New Zealand White rabbit (4.0 kg) according to the method of Kaneko *et al.* (1985). The saline solution of the antigen (250 μg/ml) was emulsified in a equal volume of Freund's complete adjuvant. The emulsion was injected into surgically exposed lymph nodes of hind limbs (400 μl) and intradermally in the back (400μl). Booster injections of immunogen were then given at intervals of 3 weeks: on each occasion 800 μL of emulsion including 400 μl of Freund's incomplete adjuvant was injected intradermally and/or subcutaneously into the rabbit's back. Blood, collected from an ear vein, was obtained 10 days after the third, fourth, and fifth booster injections. The sera (RADH I, II and III) were separated, lyophilized, and stored at -20°C prior to characterization studies.

Double Immunodiffusion Test

A double immunodiffusion test (Ouchterlony, 1953) was carried out using a plate containing 1% agarose in 0.01 M phosphate-buffered saline (pH 7.5). The ancigen (90 μg/ml) and the antisera (RADH I, II, and III: neat and diluted 1:1) were applied (15 μl of each) to the central and peripheral wells, respectively, and the plate was incubated for 2 days at 4°C.

Radioimmunoassay

Stanniocalcin was iodinated with $Na^{125}I$ (Edmonton Radiopharmaceutical Centre, University of Alberta, Edmonton, Alberta) using commercial reagents (Iodogen, Pierce Chemical Co., Rockford, IL) as previously described (Salacinski *et al.*, 1981). Iodinated

stanniocalcin was separated from free ^{125}I by gel filtration of Sephadex G-100, and its cross-reactivity with serial dilutions of hypocalcin antisera was determined. To test whether binding of the tracer to the antibody could be competitively displaced, serially diluted, unlabeled stanniocalcin was incubated with the tracer and the antibody. The specificity of the tracer binding was decided by the presence of cross-reactivity of crude extracts of coho salmon and catfish CS, of sockeye salmon teleocalcin (Wagner *et al.*, 1986), other glycoprotein hormones (rat FSH, chicken LH, bovine TSH), and peptides (salmon calcitonin, bovine PTH, bovine PTH-1-34, human angiotensin I and II). The presence of stanniocalcin-like immunoreactivity in fish plasma was also determined.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA for stanniocalcin was established using the specific antiserum (RADH I) according to the method of Engvall and Perlmann (1972) with some modifications. First, the wells of a microtitration plate (Linbro, Flow Laboratories) were coated with 200 μl of serial concentrations of stanniocalcin or unknown samples. The plate was incubated at 37^{o} for 45 min and then at 4^{o} for 18 hr. The wells were then washed four times and 200 μl of the antiserum or normal rabbit serum (1:1000-16,000) was added to each well. The plate was incubated at 37^{o} for 1 hr. The amount of alkaline phosphatase bound to the wells was determined by using p-nitrophenyl phosphate (NPP, Fisher Scientific) as a substrate. After washing the wells, 200 μl of 0.1% NPP containing 1 mM $MgCl_2$ was added, and

the plate was incubated at room temperature for 4 hr. The absorbance of each sample at 405 nm was measured using a Titertek Multiskan (Flow Laboratories).

Immunocytochemistry

Goldfish (50-100 g) were killed by decapitation and the CS were removed, together with surrounding kidney tissue. The CS were fixed with Bouin's solution (without acetic acid) for 18 hr, dehydrated in ethanol, and embedded in paraplast. Tissue sections (3 μm thickness) were then mounted on glass slides and immunocytochemically stained by the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al.*, 1981) using commercial reagents (Vectastain ABC Kit, Vector Laboratories). The sections were incubated with specific antisera (1:1000-4000) for 18 hr at 4^{o}. The specificity of the immunocytochemical staining was confirmed by preabsorbing the antiserum with the antigen (10 μg/ml antiserum at the working dilution).

RESULTS

Double Immunodiffusion Test

The antisera (RADH I, II, and III) raised against the stanniocalcin purified from the rainbow trout CS were applied to Ouchterlony's double immunodiffusion test. Each serum formed a distinct precipitin line with the antigen (Fig. II-1), whereas the control serum, which was obtained from the immunized rabbit just before the first injection, showed no reaction. Although there was no apparent difference in the immunoreaction among these three

antisera, the neat antisera consistently formed stronger precipitin lines than half-diluted ones.

Radioimmunoassay

Titration curves of antisera against stanniocalcin were obtained, as shown in Fig. II-2. The binding of the tracer of RADH I, II, and III at a final concentration of 1:4000 was 34, 28, and 24%, respectively. Under these conditions the binding of the tracer to RADH I was competitively displaced by unlabeled stanniocalcin in a sigmoidal fashion (Fig. II-3). At concentrations of 0.65, 10 and 500 ng/ml, the displacement of total binding was 15, 50, and 80%, respectively. The binding of the tracer to the antisera was also displaced by serial dilutions of teleocalcin (Fig. II-3), in a manner parallel to that of the stanniocalcin standard, although it was less (by 50%) immunoreactive (as determined at 50% binding).

Extracts of coho salmon and catfish CS (Fig. II-3), over the range 0.1-1000 μg protein/ml, produced dose-response inhibition curves parallel to the standard. Stanniocalcin-like immunoreactivity was also present in the plasma of flounder (Fig. II-3), as indicted by a dose-related displacement of binding. Dogfish plasma, however, did not cross-react in this assay.

The binding of the tracer to the antisera was not displaced by the other glycoproteins and peptides assayed, the cross-reactivity of each being less than 0.001%.

Enzyme-Linked Immunosorbent Assay (ELISA)

Serial dilutions of the antiserum were tested for detection of stanniocalcin. The results are shown in Fig. II-4. First, with increasing antiserum dilution, phosphatase activity tended to decrease slightly at all stanniocalcin concentrations tested, and higher concentrations of stanniocalcin consistently resulted in stronger phosphatase activity at any dilution of the antiserum. The standard curve obtained with the antiserum at a dilution of 1:2000 is shown in Fig. II-5. Serial concentrations of stanniocalcin produced a dose-response curve over the range 3.9-250 ng/ml. The extract of the coho salmon CS also produced dose-response curve, which was parallel to the stanniocalcin standard (Fig. II-5).

Teleocalcin, bovine PTH, and its bioactivity fragment (1-34) were checked for cross-reactivity with the antiserum against stanniocalcin in the ELISA. Teleocalcin showed a dose-response curved parallel to the standard obtained with stanniocalcin (Fig. II-5) and the cross-reactivity was estimated to be 42%. Bovine PTH and its fragment (1-34), on the other hand, failed to cross-react with the antiserum, the cross-reactivity being less than 1% (data not shown).

Immunocytochemistry

The CS of goldfish, like those of other species studied, are encapsulated, small glands. Each corpuscle in subdivided into numerous lobules by connective tissue septa. The lobules consist mainly of endocrine cells, which are arranged in a radial fashion.

The best staining was obtained with the antisera (RADH I, II and III) diluted 1:2000, whereas the specific immunoreaction was weaker at a dilution of 1:4000 and the background staining became strong at 1:1000. There was no distinct difference among those three antisera. Most gland cells showed strong immunoreaction with the antisera, while some cells located in the central region of the lobules displayed no immunoreactivity (Figs. II-6 and II-7). Furthermore, the specific immunoreaction was not observed in other regions of the CS and the surrounding kidney tissue. In the immunoreactive cells, the reaction was restricted to the cytoplasm and was not found in the nuclei. In addition, the control procedure in which the specific antisera were preabsorbed with the antigen resulted in complete extinction of the immunoreaction.

DISCUSSION

In the present study, highly specific antisera were raised against stanniocalcin, a 54-kDA glycoprotein purified from rainbow trout CS. The specificity of the antisera was determined by using Ouchterlony's double immunodiffusion test, RIA, ELISA, and immunocytochemistry.

Although several groups have purified hypocalcemic factors from teleost CS (e.g. Ma and Copp, 1987; Wagner *et al.*, 1986; Lafeber *et al.*, 1987), the results of the present study indicate that these glycoproteins are immunologically related, despite their different molecular size. While stanniocalcin used in the present study for antiserum generation had a molecular weight of 54 kDA,

salmon teleocalcin was reported to be a 39.3-kDa product (Wagner *et al.*, 1986). However, it is possible that teleocalcin represents a fragment of the intact stanniocalcin molecule, since our immunological tests showed cross-reactivity of teleocalcin with the antiserum against stanniocalcin. Lafeber *et al.* (1987) isolated a 41-kDa product from the rainbow trout CS, which was thought to be a fragment of the native 54-kDa stanniocalcin. This 41-kDa stanniocalcin fragment may be equivalent to the salmon 39.3-kDa teleocalcin.

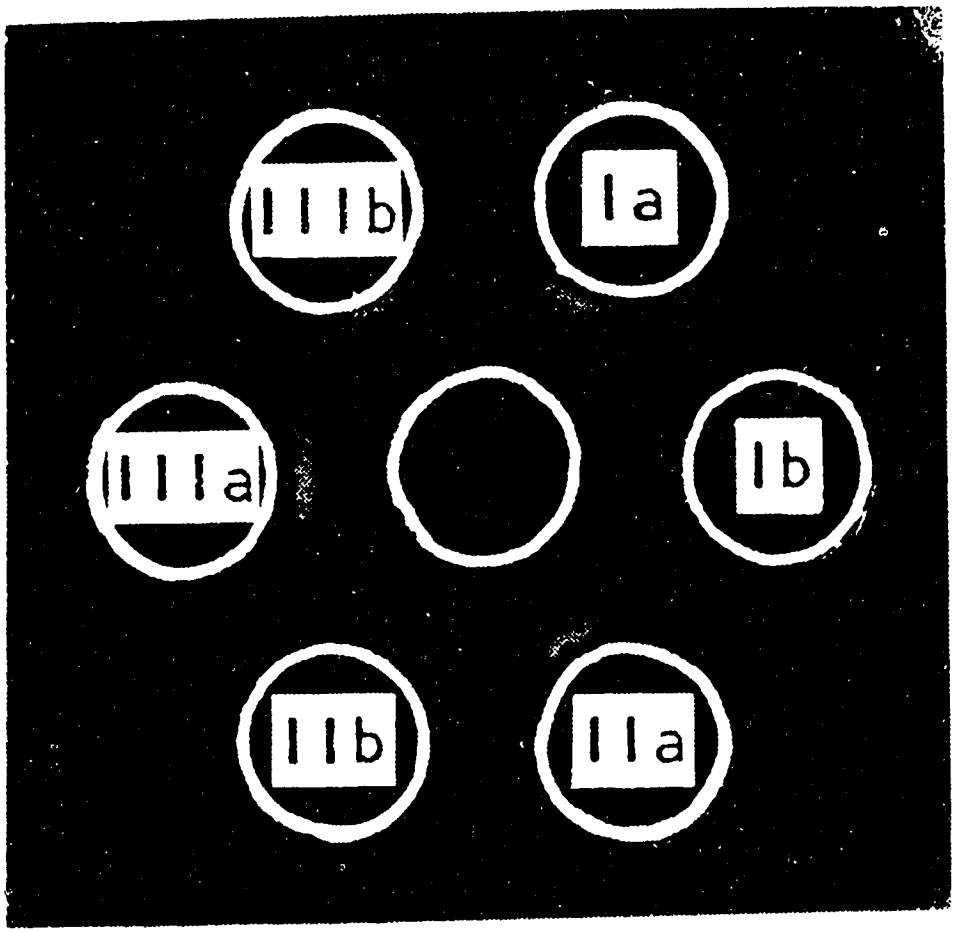
In addition to its similarity to teleocalcin, stanniocalcin has also been suggested to be immunologically related to PTH, since the extracts of the CS show PTH immunoreactivity (Milet *et al.*, 1980, 1982; Harvey *et al.*, 1987) and the endocrine cells in the CS react immunocytochemically with anti-PTH serum (Lopez *et al.*, 1981, 1982, 1984a, b). In the present study, however, bovine PTH did not cross-react with the antiserum against stanniocalcin in the RIA and the ELISA. This is somewhat surprising, especially because Lafeber *et al.* (1986) showed that the CS product of rainbow trout possessed PTH-like bioactivity when tested in a PTH bioassay involving bone resorption in embryonic mouse calvaria. These results therefore suggest that stanniocalcin and PTH share some structural similarities, although they are not identical.

The presence of two types of cells has been shown in the CS of various species (for review see Wendelaar Bonga and Pang, 1986) including goldfish (Oguri, 1966; Ogawa, 1976; Wendelaar Bonga *et al.*, 1980). One cell type is more abundant and contains numerous

large secretory granules (type 1), another is characterized by the presence of small secretory granules (type 2). However, it still remains unclear whether these two cell types reflect different physiological conditions of a single cell type, or represent functionally different cells. In the present immunocytochemical study at the light microscopical level, the immunoreaction was observed in most but not all endocrine cells in the goldfish CS. It is likely, on the basis of the morphology of the CS, that the immunoreactive and nonimmunoreactive cells correspond to the type 1 and type 2 cells, respectively. Thus, the predominant type 1 cells appear to be the source of stanniocalcin in goldfish CS. This is in agreement with findings by Cohen *et al.* (1975), Wendelaar Bonga *et al.* (1976, 1980), Meats *et al.* (1978), and Aida *et al.* (1980a, 1980b). Nevertheless, there still remains the possibility that the type 2 cells might fail to be immunocytochemically stained, simply because the stanniocalcin content was too small to be detected by immunocytochemistry.

In the RIA study the immunoreactive stanniocalcin was found in the plasma of flounder, but not in that of dogfish. The CS are believed to exist in holostean and teleostean fish, but are not found in other vertebrates (Wendelaar Bonga and Pang, 1986). The finding of immunoreactive stanniocalcin in the plasma of flounder and its absence in dogfish plasma may support this hypothesis, although it is possible that a stanniocalcin-like substance is present in dogfish but sufficiently different from that of rainbow trout not to cross-react with the antisera used.

Figure II-1 Ouchterlony's double immunodiffusion test with stanniocalcin (a central well) and rabbit antisera (peripheral wells) raised against stanniocalcin I, RADH-I; II, RADH-II; III, RADH-III; a, neat; b, diluted 1:1.


IIIb
Ia
IIIa
Ib
IIb
IIa

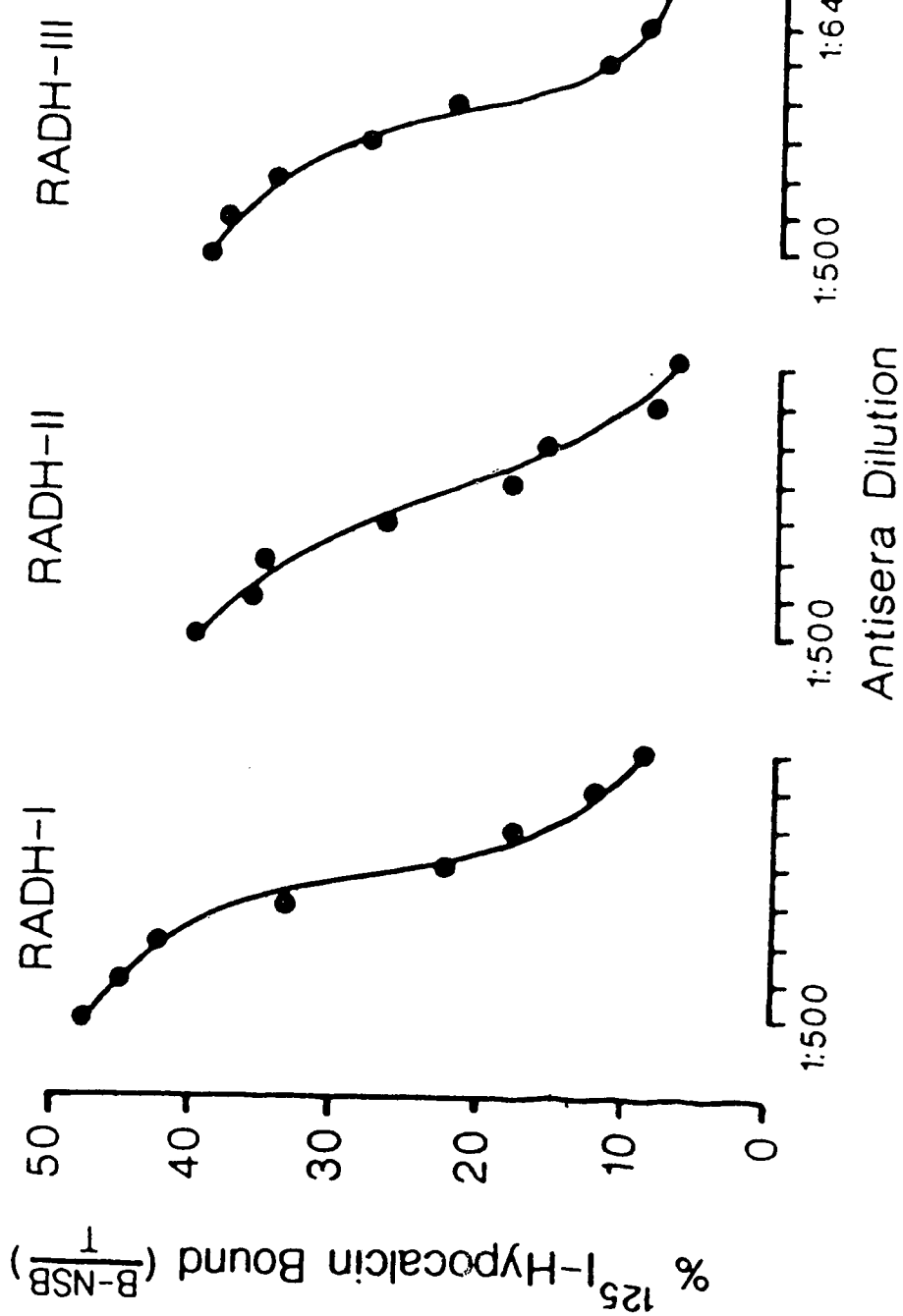


Figure II-2 Titration curves of rabbit antisera (RADH-I, II, and III) raised against stanniocalcin.

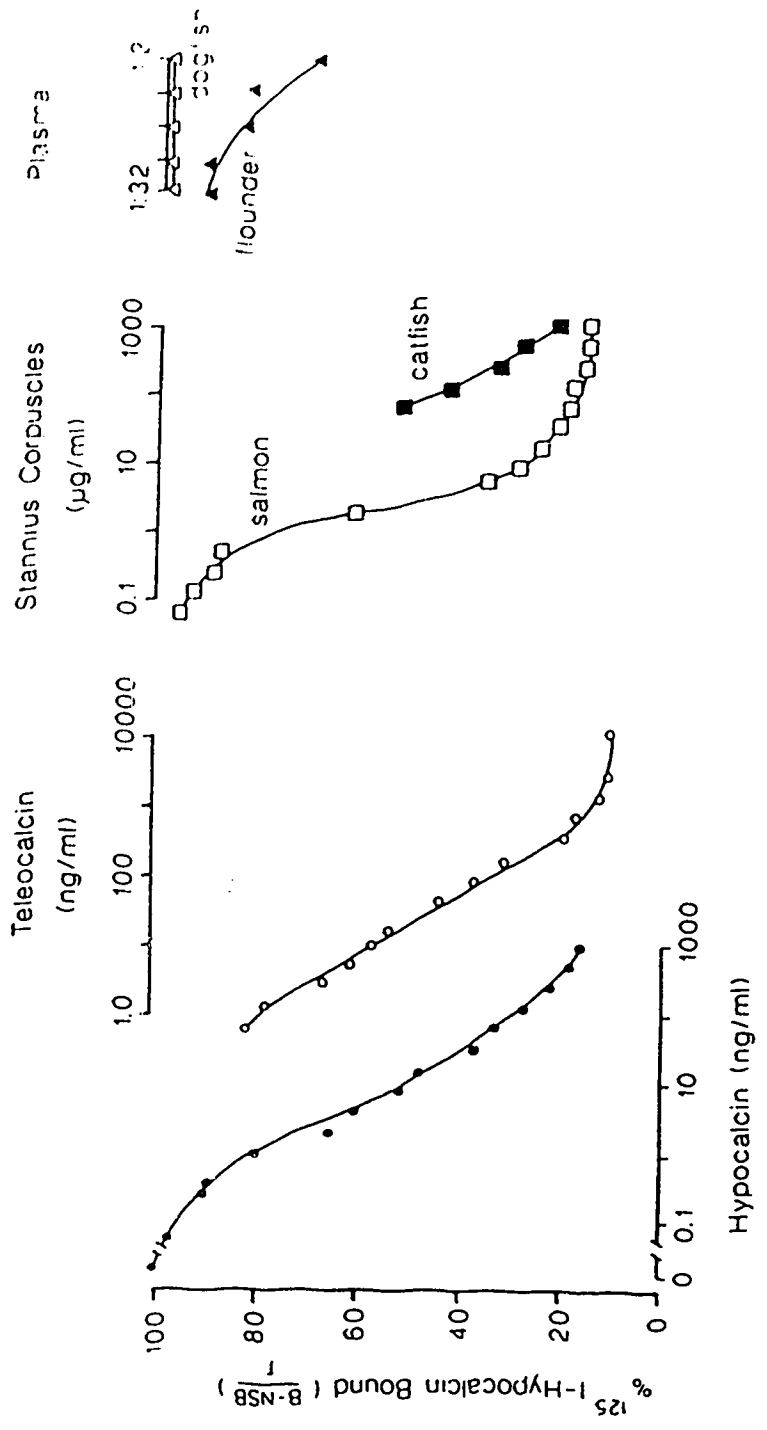


Figure II-3 Inhibition of binding of ^{125}I-labelled stanniocalcin to the antiserum (RADH-I) by stanniocalcin, teleocalcin, extracts of coho salmon and catfish CS, and plasma of flounder and dogfish.

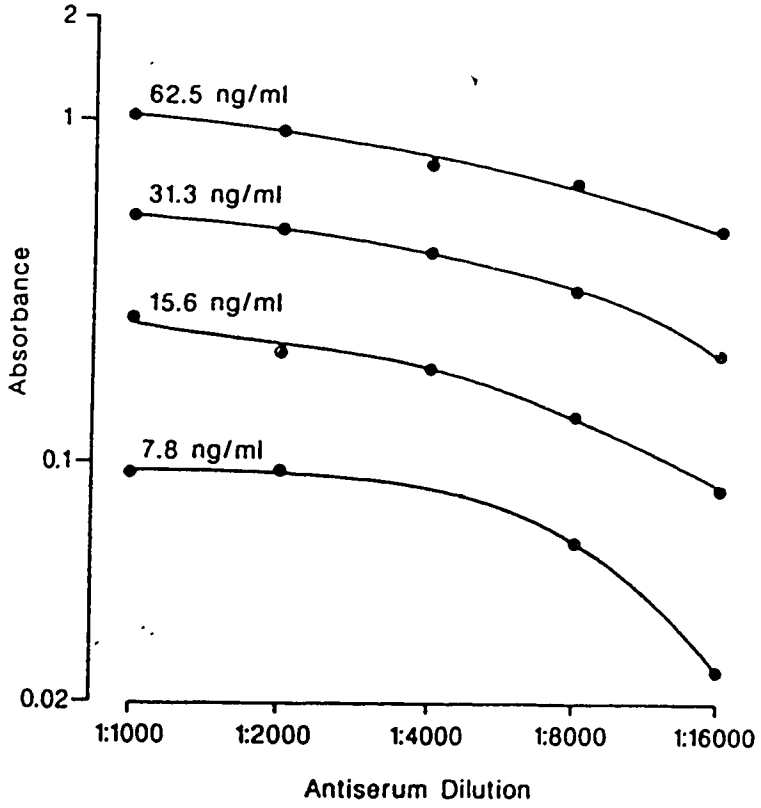


Figure II-4. Influence of dilution of the antiserum (RADH-I) on detection of stanniocalcin in ELISA.

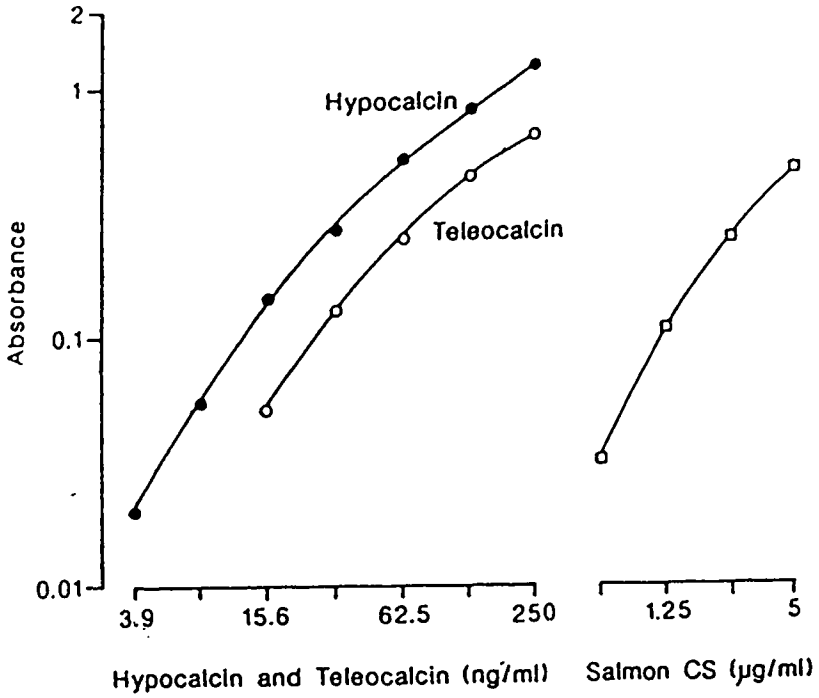
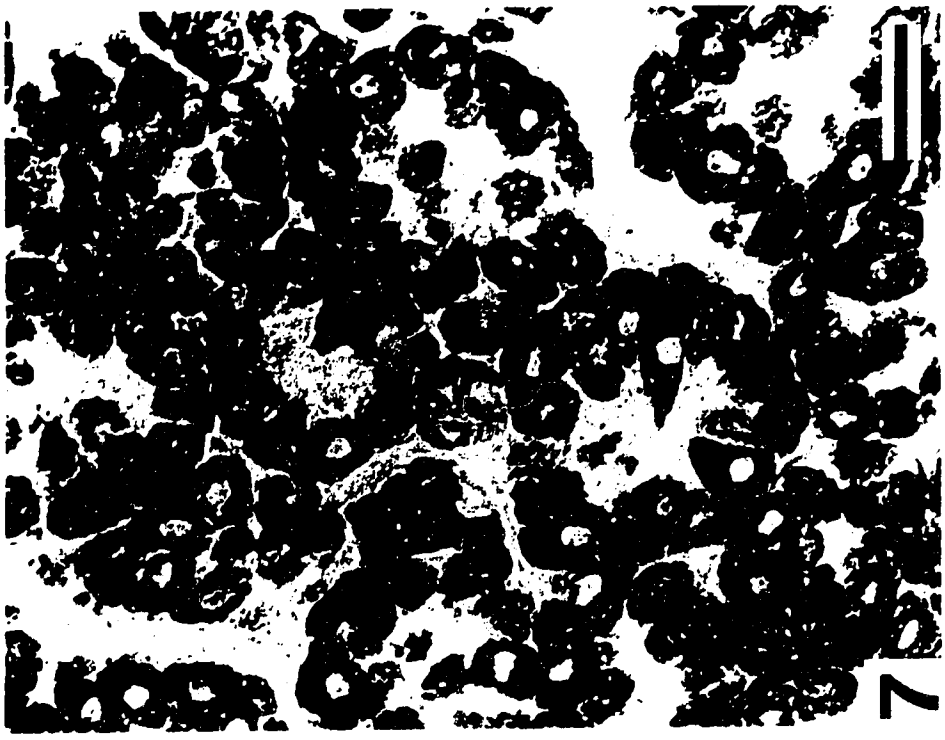


Figure II-5. Dose-response curves produced by stanniocalcin, teleocalcin, and coho salmon CS extract in ELISA.

Figure II-6. Goldfish CS stained with the antiserum (RADH-I) at a dilution of 1:2000. Scale, 50 μm.

Figure II-7. Higher magnification of goldfish CS stained with the antiserum (RADH-I). Scale 10 μm.



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C H A P T E R J I I

HYPO- AND HYPERCALCEMIC PEPTIDES IN FISH PITUITARY GLANDS[1]

1. A version of this chapter is in press. Fraser RA, Kaneko T, Pang PKT, Harvey S, 1990 Hypo- and hypercalcemic peptides in fish pituitary glands. Am J Physiol.

INTRODUCTION

Fish lack encapsulated parathyroid glands (Pang and Pang, 1986) but peptides with parathyroid hormone (PTH) immunoreactivity (IR) are present in fish plasma (Harvey *et al.*, 1987) and may originate from the pituitary gland (Fenwick, 1982a; Harvey *et al.*, 1987; Pang and Pang, 1986; Parsons *et al.*, 1978) or the corpuscles of Stannius (CS) (Fenwick 1982b; Harvey *et al.*, 1987; Lopez *et al.*, 1984; Milet *et al.*, 1982; Milet *et al.*, 1989). Both of these glands participate in the calcium regulation of teleosts. The pituitary increases serum calcium levels in response to hypocalcemia, while the corpuscles of Stannius lower serum calcium levels in response to hypercalcemia (Pang and Pang, 1986).

Although PTH stimulates increases in serum calcium levels in mammals it has been reported to lower (under certain conditions) serum calcium levels in two species of fish (Wendelaar Bonga *et al.*, 1986). IR PTH-like peptides have been located in the corpuscles of Stannius, which are also the source of a hypocalcemic hormone. This hormone (stanniocalcin) is structurally and immunologically distinct from PTH (Harvey *et al.*, 1987; Lopez *et al.*, 1984; Milet *et al.*, 1982; Milet *et al.*, 1989). The possibility that stanniocalcin may similarly be present in the fish pituitary has therefore been investigated in the present study. In addition, since PTH and another hypercalcemic peptide, PTH related protein (PTHrp) (Moseley *et al.*, 1987; Suva *et al.*, 1987), recently have been detected in the mammalian brain (Pang *et al.*, 1988a; Pang *et al.*, 1988b; Weir *et al.*, 1990), the

possibility that PTHrp may also be present in the fish pituitary has also been examined.

MATERIALS AND METHODS

Tissue Extraction

Freshly caught Coho salmon (*Oncorhynchus kisutch*) were decapitated and the pituitary glands were rapidly dissected from the heads prior to lyophilization and storage at -20^{0}C. The glands were then extracted in 0.1 M HCl and the supernatant was boiled and dialysed as previously described (Harvey *et al.*, 1987). For comparative purposes, skeletal muscle from decapitated rats and corpuscles of Stannius from trout were also similarly extracted. The protein content of the extracts was determined by the Bradford (1976) method, using commercial reagents (Sigma Chemical Company, St. Louis, MO).

Radioimmunoassay

The cross-reactivity of serial dilutions of the tissue extracts in specific double-antibody radioimmunoassays (RIAs) for PTH, PTHrp and stanniocalcin was determined. PTH IR was measured using a commercial antisera raised against bovine PTH-(1-84) [bPTH-(1-84), Diagnostic Systems Laboratories, Webster, TX], that was specific for the 48-64 region of the peptide (Harvey *et al.*, 1987). Bovine PTH-(1-84) was also used as the standard and the ^{125}I-labeled radioligand (Diagnostic Systems Laboratories, Webster, TX). The cross reactivity of the extract with an antiserum raised against human (h)PTH (1-34) (Cantley *et al.*, 1985) was also determined in an

RIA in which hPTH (1-34) Nle ^{8,18} Tyr^{34} (Penninsula Laboratories, Belmont, CA) was used as the standard and ^{125}I- labeled radioligand (Harvey *et al.*, 1987). PTHrp IR was determined using an antiserum raised against hPTHrp-(1-34) (Suva *et al.*, 1987), which had no cross reactivity with authentic PTH. Human PTHrp-(1-34) Tyr^{34} (Bachem Inc., Torrance, CA.) was used as the standard and was labeled with ^{125}I NaI (Amersham Corporation, Oakville, Ontario), by the iodogen method (Salacinski *et al.*, 1981). When used at a final dilution of 1:5000 this antiserum bound 20% of the radioligand and had a sensitivity of <2.0 pg/ml. The cross-reactivity of the extracts with an antiserum raised against stanniocalcin (Kaneko *et al.*, 1988) was also examined, using ^{125}I-labeled stanniocalcin (Lafeber *et al.*, 1988) as the radioligand and (because of the limited availability of pure peptide) an acid extract (0.1 M acetic acid, 10:1 vol:wt) of trout CS (Kaneko *et al.*, 1988) as the standard.

The specificities of these RIAs was demonstrated by the minimal cross reactivity of the respective standards in heterologous assays, as indicated in Table III-1.

HPLC Fractionation

Salmon pituitary extracts were defatted with n-hexane (Keutmann *et al.*, 1974), dissolved in 0.1% trifluoracetic acid (TFA) and subjected to reverse phase high performance liquid chromatography (HPLC) on a 250 X 10 mm C_{18} column (Synchron, Linden, IN), with a linear gradient of 0-80% acetonitrile, containing 0.1% TFA, at a flow rate of 4 ml/min. The fractions (4 ml) were monitored by ultraviolet

detection at a wavelength of 280 nm and compared with the elution profiles of synthetic hPTH-(1-84) (Sigma Chemical Co., St. Louis, MO) and isolated stanniocalcin (Lafeber *et al.*, 1988) chromatographed under the same conditions. Freeze-dried fractions were assayed for PTH-(1-34), PTH-(1-84), PTHrp-(1-34) and stanniocalcin by their respective RIA's.

Immunocytochemistry

The heads of decapitated platyfish (*Xiphophorus maculatus*) were fixed in Bouin's solution without acetic acid for 18 h. After decalcification in 5% trichloroacetic acid and dehydration in ethanol, the brain and pituitaries were embedded in paraplast. Sagittal sections (3 μm) were mounted on gelatin-treated slides and then stained by the avidin-biotin complex (ABC) method of Hsu *et al.* (Hsu *et al.*, 1981), using commercial reagents (Vectastain ABC Kit, Vector Laboratories). The sections were incubated for 18 h at 4^0C with highly specific antisera raised against stanniocalcin (Kaneko *et al.*, 1988) and PTHrp (Suva *et al.*, 1987) at dilutions of 1:1000 and 1:400 respectively.

RESULTS

Radioimmunoassay

As expected, serial dilutions of the salmon pituitary extract displaced the binding of ^{125}I-PTH-(1-84) and ^{125}I-PTH-(1-34) $Nle^{8,18}Tyr^{34}$ to antisera raised against bPTH-(1-84) or bPTH-(1-34), in a manner parallel to the respective standards (Figs. 1a and 1b). The estimated PTH-(1-84) and PTH-(1-34) IR of the extract

determined from the concentrations inducing 50% inhibition of radioligand binding were 0.5 and 6.25 ng/mg protein, respectively. Dilutions of the pituitary extract also displaced the binding of ^{125}I-hPTHrp-(1-34) to hPTHrp-(1-34) antisera, in a manner parallel to the hPTHrp-(1-34) standard, (Fig. III-1c) and had an IR-hPTHrp-(1-34) content of 2.5 ng/mg protein. Serial dilution of the extract also displaced ^{125}I- stanniocalcin binding to its antisera (Fig. III-1d), in a concentration-dependent manner. The IR stanniocalcin content of the extract was equivalent of 0.66 ng CS protein/mg protein. Extracts of rat skeletal muscle had no IR in any of the RIAs tested (Figs. III-1a-d), extracts of trout CS had IR only in the stanniocalcin assay (Figs III a-d).

HPLC Fractionation

Multiple protein fractions were separated from the pituitary extract after C_{18} purification (Fig. III-2). Protein fractions 32-38, that eluted ahead of hPTH-(1-84) (fraction 39), had bPTH-(1-84) immunoreactivity (2.4 ng bPTH-(1-84)/mg protein), whereas other fractions were without IR in the bPTH-(48-64) RIA (Fig. III-2). A single protein fraction (fraction 39), with an IR PTH-(1-34) content of 13.2 ng/mg protein co-eluted with authentic hPTH-(1-84) (Fig. III-2). Pituitary fractions that eluted with PTHrp-(1-34) (Moseley *et al.*, 1987) (fraction 35-37 Fig. III-2) or stanniocalcin (fraction 52, Fig. III-2) were also separated, with concentrations of 100 ng/mg protein and 405.2 ng/mg protein in their

respective RIAs. All of the other fractions were devoid of PTH (1-34), PTHrp-(1-34) or stanniocalcin IR.

Immunocytochemistry

When platyfish brain and pituitary sections were exposed to hPTHrp-(1-34) antisera, no immunocytochemical staining was observed (data not shown). However, using antisera raised against stanniocalcin, specific immunoreactivity was detected in the brain and pituitary gland (Fig. III-3). The immunoreaction detected in the pituitary was mainly located in the neuro-pars intermedia, and in nerve fibres in the pars distalis (Fig. III-3a), although no adenohyopophyseal cells were immunoreactive. In the brain, immunoreactive cell bodies were found in the nucleus preopticus (NPO) (Fig. III-3b). The immunoreaction was restricted to the cytoplasm of the cells in both the posterodorsal part (*pars magnocellularis*) and anteroventral part (*pars parvocellularis*) of the NPO. Preabsorption of the antisera with stanniocalcin eliminated staining (data not shown).

DISCUSSION

These results clearly demonstrate the presence of calcium-regulating hormones in fish pituitary glands.

While IR-PTH has been found previously in extracts of the Coho salmon pituitary gland and located by immunohistochemistry in the goldfish pituitary (Harvey *et al.*, 1987; Kaneko and Pang, 1987), these results show that this immunoreactivity co-elutes with authentic PTH-(1-84) and is dissimilar to PTHrp or stanniocalcin. The

cross-reactivity of tissue extracts with antisera directed against both the N-terminus and mid-molecule of PTH (1-84) provides strong evidence for the presence of this peptide in the Coho salmon pituitary gland. The detection of a single IR protein peak using the hPTH-(1-34) RIA and a broader, multiple peak using the bPTH-(48-64) RIA is also consistent with the existence of PTH-(1-84) in the tissue extracts, since C-terminal degradation products (Harvey *et al.*, 1987) will not be detected by the hPTH-(1-34) RIA.

In addition to PTH, PTHrp or a PTHrp-like peptide would also appear to be present in extracts of the salmon pituitary gland. Although PTHrp immunoreactivity could not be detected by immunocytochemistry, this may reflect the poorer sensitivity of this technique or changes in the immunoreactive epitope or loss of peptide when the tissue is dehydrated and fixed in paraplast sections.

Parathyroid hormone-related protein was originally isolated from a human lung cancer cell line (Moseley *et al.*, 1987; Suva *et al.*, 1987) but has since been shown to be expressed in a variety of normal tissues (Goltzman *et al.*, 1989), including the rat brain (Weir *et al.*, 1990). It has not, however, been demonstrated previously in the pituitary gland or in species other than mammals. The presence of PTHrp IR in the salmon pituitary gland therefore suggests an early evolutionary divergence of the PTH and PTHrp genes, in accordance with the hypothesis previously proposed on the basis of the sequence homology of PTH and PTHrp and because of the chromosomal localization and structural organization of their genes (Goltzman *et al.*, 1989).

In mammals PTHrp has been shown to have discrete actions related to cellular growth and differentiation (Holick *et al.*, 1988; Insogna *et al.*, 1989), although it interacts with PTH receptors (Abou-Samra *et al.*, 1989) and can induce hypercalcemia by actions at renal and skeletal sites (Goltzman *et al.*, 1989). Since PTHrp or a PTHrp-like peptide is present in the fish pituitary gland, it may also have peripheral effects in piscine calcium regulation (Pang and Pang, 1986).

Stanniocalcin, distinct from PTH-like peptides, has been purified from the corpuscles of Stannius (Butkus *et al.*, 1987; Harvey *et al.*, 1987; Kaneko *et al.*, 1988; Lafeber *et al.*, 1988; Milet *et al.*, 1989; Wagner *et al.*, 1986) and exerts hypocalcemic actions (Wagner *et al.*, 1988). The results of the present study show that stanniocalcin or a stanniocalcin-like peptide is also present in nerve fibres in the platyfish neuro-pars intermedia but it is not present in the adenohypophysis. Immunoreactive stanniocalcin is also located in the brain, in cell bodies in the pars magnocellularis and pars parvocellularis of the NPO. The distribution of IR stanniocalcin in the platyfish brain and pituitary gland is, therefore, strikingly similar to that of IR-PTH (Kaneko *et al.*, 1988; Kaneko and Pang, 1987) and both may constitute novel hypophysiotropic neurosecretory systems. This hypothesis is supported by the demonstration of peptidergic neurons with PTH IR that terminate around hypophysial blood vessels in the median eminence of mammals (Pang *et al.*, 1988b). Indeed, since IR-PTH and IR-stanniocalcin are also present in the neural ganglia of

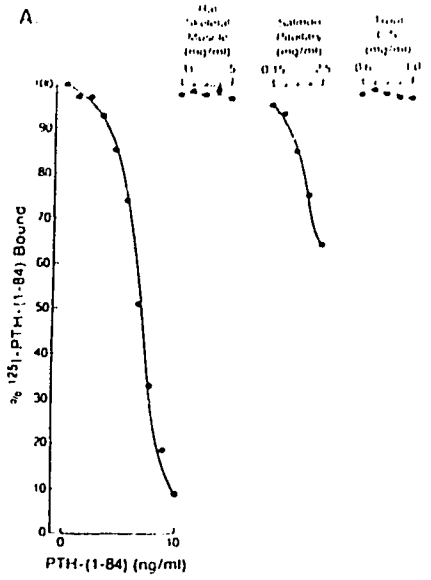
invertebrates (Wendelaar Bonga *et al.*, 1989), a neural or neuroendocrine role may have been the ancestral function of these peptides.

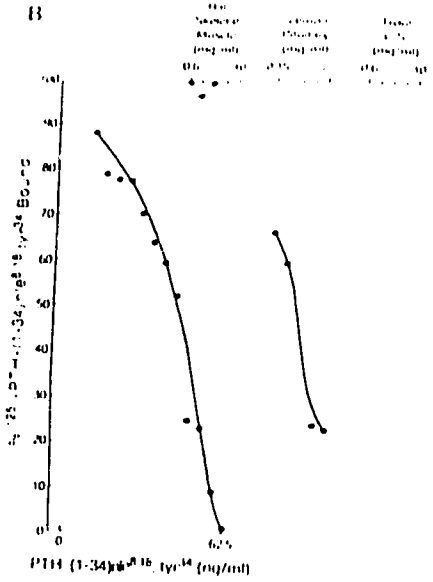
In summary, these results demonstrate for the first time the presence of PTH-, PTHrp- and stanniocalcin-like peptides in the fish pituitary gland using specific antibodies and suggest roles for these peptides in neuroendocrine regulation.

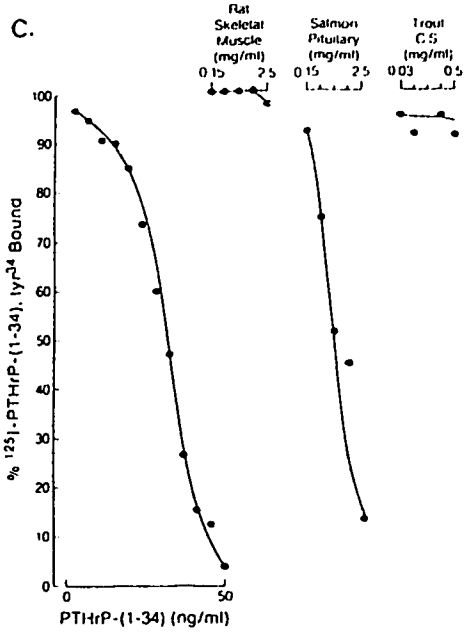
Table III-1 Percent Cross Reactivity of RIAs with Tested Antigens

RIA/Peptide	tStanniocalcin	bPTH-(1-84)	hPTH-(1-34) $nle^{8,18}$ tyr^{34}	hPTH-(1-34)	hPTHrP-(1-34)
tStanniocalcin	80%	<0.01%	<0.01%	<0.01%	<0.01%
hPTH-(48-53)	<0.01%	100%	<0.01%	<0.01%	<0.01%
rPTH-(1-34)	<0.01%	98%	97%	100%	<0.01%
hPTHrp-(1-34)	<0.01%	<0.01%	<0.01%	<0.01%	<89%

Figure III-1. Cross-reaction of boiled dialysed extracts of salmon pituitaries trout CS, and rat skeletal muscle in radioimmunoassays (RIAs) for: a) PTH-(1-84), b) PTH-(1-34), c) PTHrp-(1-34), d) stanniocalcin.


A
% 125I-PTH-(1-84) Bound
PTH-(1-84) (ng/ml)


B


C.
Rat Skeletal Muscle (mg/ml)
Salmon Pituitary (mg/ml)
Trout CS (mg/ml)
% 125I-PTHrP-(1-34), tyr34 Bound
PTHrP-(1-34) (ng/ml)

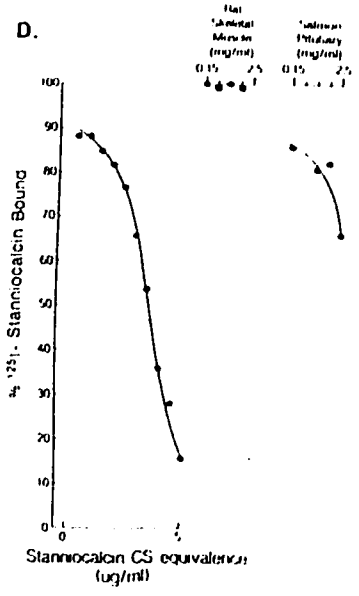

D.
% 125I- Stanniocalcin Bound
Stanniocalcin CS equivalents
(ug/ml)

Figure III-2. Elution profile of salmon pituitary extract separated by reverse-phase HPLC in a 0-80% acetonitrile gradient. Fractions were radioimmunoassayed for the presence of stanniocalcin, PTHrp-(1-34), PTH-(1-34) and PTH-(48-62). Immunoreactive fractions were compared to the elution of: A) hPTH-(1-84) and B) stanniocalcin.

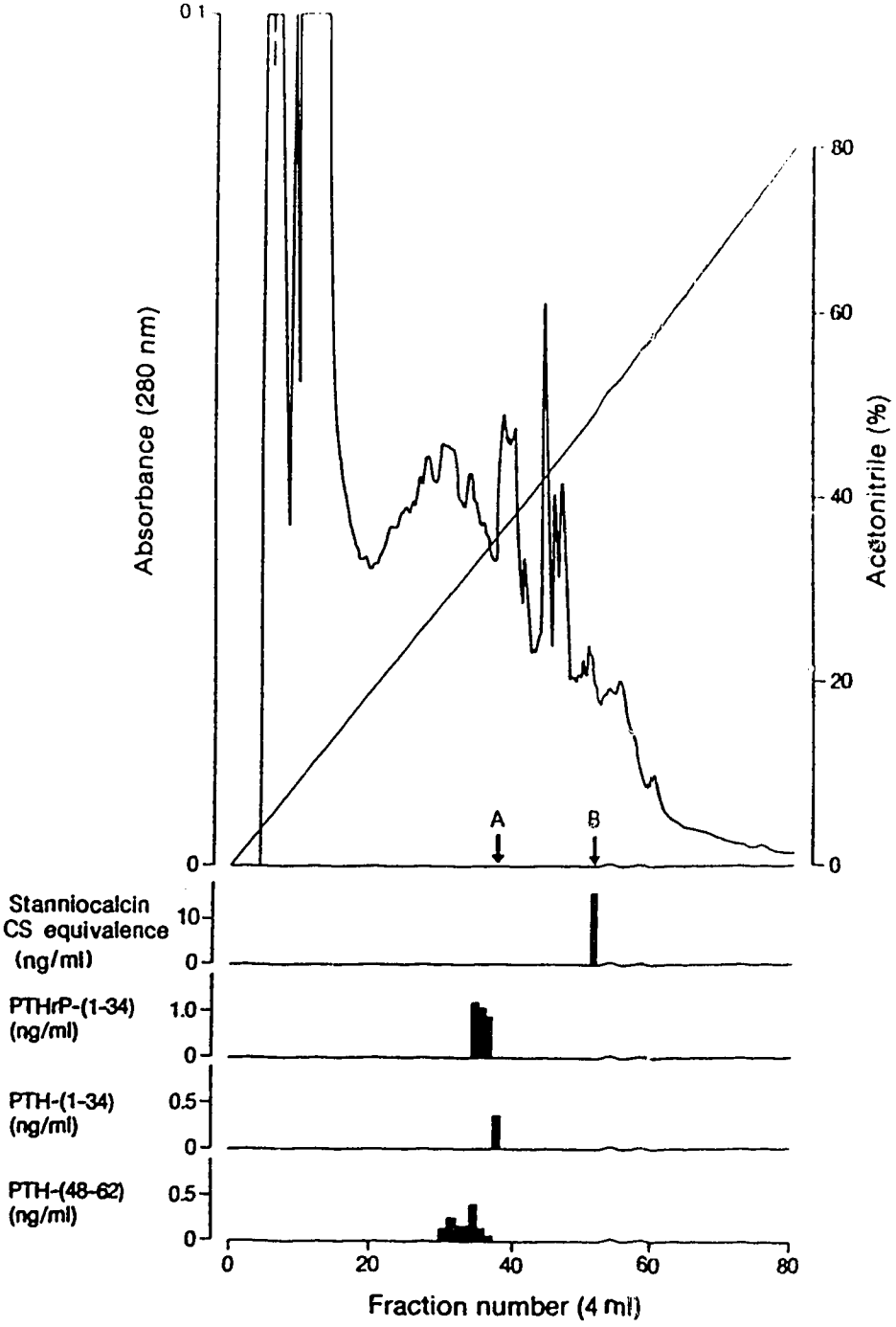
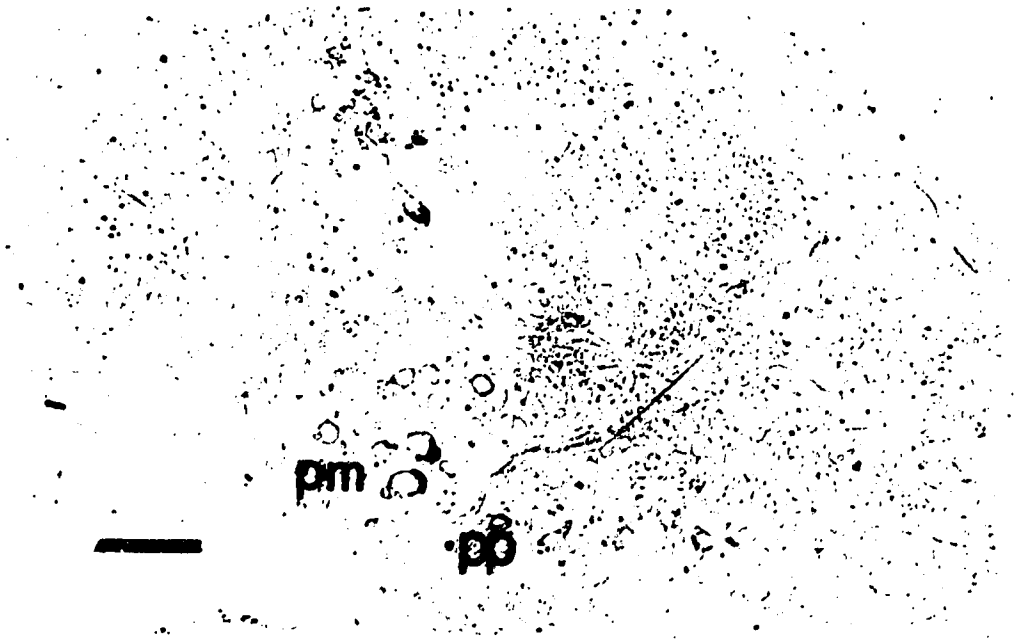

0 1
Absorbance (280 nm)
0
80
60
40
20
0
Acetonitrile (%)
A
B
Stanniocalcin
CS equivalence
(ng/ml)
10
0
PTHrP-(1-34)
(ng/ml)
1.0
0
PTH-(1-34)
(ng/ml)
0.5
0
PTH-(48-62)
(ng/ml)
0.5
0
0
20
40
60
80
Fraction number (4 ml)

Figure III-3A. Sagittal section of the platyfish pituitary showing stanniocalcin-like immunoreactivity. PD, *pars distalis*; PI, *pars intermedia*. Anterior to right. X150. Bar=50μm

Figure III-3B. The nucleus preopticus (NPO) in the platyfish brain showing cell bodies with stanniocalcin-like immunoreactivity. pm, *pars magnocellularis*; pp, *pars parvocellularis*. Anterior to right. X150. Bar=50μm

A



B

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CHAPTER IV

PARATHYROID HORMONE-LIKE IMMUNOREACTIVITY IN NEURAL TISSUES OF TETRAPOD VERTEBRATES[1]

1. A version of this chapter has been published. Pang PKT, Harvey S, Fraser R, Kaneko T, 1988 Parathyroid hormone-like immunoreactivity in brains of tetrapod vertebrates. Am J Physiol 255 (Regulatory Integrative Comp Physiol 24): R635-R642.

INTRODUCTION

The occurrence of many ectopic hormone syndromes has been demonstrated by the ubiquitous distribution of immunoassayable polypeptide hormones in endocrine and nonendocrine tissues (Kendall and Orwoll, 1980; Said, 1980; Sherwood, 1979). Parathyroid hormone (PTH), in particular, is not only synthesized and secreted by the parathyroid glands but also by malignant tumors in a variety of extraparathyroidal sites (Minne and Ziegler, 1983; Rabbani *et al.*, 1986). Immunoreactive (IR) PTH has also been found in human cerebrospinal fluid (Balabanova *et al.*, 1984), and the brain and pituitary gland of sheep have been shown to be extraparathyroidal sites of IR PTH production (Balabanova *et al.*, 1985; Balabanova *et al.*, 1986b). Because we have also identified IR PTH in the brain and pituitary gland of fish (Harvey *et al.*, 1987), which lack encapsulated parathyroid glands (Pang *et al.*, 1982), the occurrence of PTH-like peptides in the brain may have evolutionary significance. The possibility that brain IR PTH is also present in other vertebrate taxa has therefore been investigated in the present study.

MATERIALS AND METHODS

Immunoreactive PTH was determined in the plasma and tissues of amphibian (mud puppies, *Necturus maculosus*; marine toads, *Bufo marinus*: bullfrogs, *Rana catesbeiana*), reptilian (painted turtle, *Chrysemys picta;* garter snakes, *Thamnophis sirtalis*), avian (Japanese quail, *Coturnix coturnix japonica;* domestic fowl.

Gallus domesticus; domestic ducks, *Anas platyrhynchos domesticus*) and mammalian (European rabbits, *Oryctolagues cuniculus*; black rats, *Rattus rattus;* golden hamsters, *Mesocricetus auratus*; house mice, *Mus musculus; and guinea pig*) species. All the animals, adults of either sex, were obtained from commercial suppliers and were provided with food and water *ad libitum* before autopsy. To determine the influence of dietary calcium status of plasma and tissue IR PTH concentrations, groups of rats (n=12) were provided with diets (obtained from Teklad Diets, Madison, WI) containing 0.0, 0.5, or 2.5% calcium (diets TD 81274, TD 83028 and TD 83467, respectively) for 10 days before death.

Heparinized blood samples were obtained from each animal after decapitation or cardiac puncture, and individual or pooled aliquots of the plasma samples from each species were stored at -20^{o}C before analysis. At autopsy, brain, hypothalamus, whole pituitary glands, myenteric plexus, liver, kidney, and muscle tissue were collected from each animal and rapidly frozen. The tissues were subsequently extracted (10 ml/g) in physiological saline or dilute acid (0.1 M HCl), and some of the extracts were boiled (at 96^{0}C) to destroy protease enzymes, dialyzed against distilled water (in 6,000- to 8,000-mol wt cut-off tubing), and lyophilized or subjected to separation of Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA), as previously described (Harvey *et al.*, 1987). Briefly, the extracts were applied to Sep-Pak cartridges equilibrated with 4% acetic acid and were eluted with a 90% methanol-4% acetic acid solvent, in a volume of 2 ml. The eluate was then air-dried and

redissolved in distilled water before assay. A similar procedure has been used to purify neurohypophyseal hormones from plasma (Crofton *et al.*, 1980) and for the purification of labeled PTH ((Schmidt *et al.*, 1986), and in preliminary studies it was established that other proteins (including bovine serum albumin) were separated under these conditions. Extracts of rat hypothalamus and pituitary gland were also defatted with n-hexane (Keutmann *et al.*, 1974), dissolved in 0.1% trifluoroacetic acid, and subjected to reverse-phase high-performance liquid chromatography (HPLC) on a 250 x 10 mm C_{18} column (Synchrom, Linden, IN) with a linear gradient of 0-80% acetonitrile, at a flow rate of 4 ml/min. The fractions were monitored by ultraviolet detection at a wavelength of 280 nm and compared with the elution profile of synthetic human PTH-(1-84) [hPTH-(1-84), Sigma Chemical, St. Louis, MO] chromatographed under the same conditions. Immunoreactive PTH fractions from the rat hypothalamus were combined and re-chromatographed on the same column with a reduced acetonitrile gradient slope.

Concentrations of IR PTH in the plasma and tissue extracts were determined by COOH-terminal PTH radioimmunoassays developed by Cooper *et al.* (1978) and by Diagnostic Systems Laboratories (DSL), Webster, TX (product no. 0100). Aprotinin (Sigma Chemical) was added to the radioimmunoassy buffers (at a concentration of 500 kIU/ml) to inhibit any protease activity in the plasmas or tissue extracts. The antisera used were raised in guinea pigs against bovine PTH-(1-84) and were both specific for the (48-64) region of the molecule, since

hPTH-(1-34), hPTH-(1-38), hPTH-(1-44), hPTH-(13-34), hPTH-(28-48), hPTH-(64-84), and hPTH-(70-84) (obtained from Bachem, Torrence, CA) had no cross-reactivity in either assay system, whereas hPTH-(48-64) (Bachem, Torrence, CA) had 7.37-fold and 25.0-fold more immunoreactivity than hPTH-(1-84) when assayed with the antisera raised by Cooper *et al.* (1978) or supplied by DSL, respectively. The cross-reactivity of hPTH-(53-84) with these antisera was 1.41-fold greater than that of hPTH-(48-64) when assayed with the DSL antisera but it was only 16% as immunoreactive as hPTH-(48-64) when assayed with the other antisera. The DSL antiserum [anti-PTH-(48-64) (53-64)] would thus appear to principally react with the 53-64 region of the intact PTH molecule, whereas the other antiserum [anti-PTH-(48-64), (48-53)] would appear to be primarily directed against the (48-53) region. In both assays the standard and tracer used were bovine PTH-(1-84) and ^{125}I-bPTH-(1-84), supplied by DSL. In both assays the total binding of the tracer was 30-35%, and the nonspecific binding was <4%. Both assays were specific and showed no cross-reaction (<0.001%) with anterior (growth hormone, prolactin, luteinizing hormone, follicle-stimulating hormone, thyrotrophin, adrenocorticotropic hormone, melanocyte-stimulating hormone) or posterior (arginine vasopressin, arginine vasotocin, isotocin, mesotocin, oxytocin) pituitary hormones (Harvey *et al.*, 1987), nor with angiotensin I, angiotensin II, angiotensin III, calcitonin, somatostatin$_{14}$, growth hormone-releasing factor, corticotrophin-releasing factor, thyrotrophin-releasing hormone, gonadotropin-releasing factor, melanin-concentrating hormone,

substance P, secretin, cholecystokinin, gastrin, insulin, glucagon, vasoactive intestinal peptide, or peptide histidine isoleucine (Peninsula Laboratories, Belmont, CA). The minimum detectable doses of IR PTH in the PTH-(48-64), (53-64) and PTH- (48-64), (48-53) radioimmunoassays were 0.10 and 0.15 ng/ml, respectively. Concentrations of plasma ionized calcium were determined using an *in vitro* Stat Electrolyte Analyzer (Ionetics, CA), and concentrations of tissue protein were determined according to the method described by Bradford (1976). Statistical differences on the results were determined by analysis of variance (ANOVA).

RESULTS

Serial dilutions of amphibian, reptilian, avian, or mammalian plasma displaced the binding of ^{125}I-PTH-(1-84) to PTH-(48-64), (53-64) antisera in a manner parallel to that induced by the PTH standard (Fig. IV-1). The maximum displacement of binding by a pool of garter snake plasma was only 10%, but for all other species a 30-50% displacement of binding was observed with undiluted plasma pools. Similar dose-response inhibition curves for the displacement of tracer binding to the PTH-(48-64), (48-53) antisera were also observed with the same plasma pools (data not shown). For each species, the mean plasma PTH concentration determined by both antisera ranged between 0.2 and 0.5 ng/ml (Table IV-1).

In addition to the plasma, crude saline extracts of the pituitary, brain, liver, kidney, or muscle tissue of each species also displaced the binding of the PTH tracer to the PTH-(48-64),

(53-64) antisera (Fig. IV-2). However, after boiling and dialysis of the extracts, PTH-like immunoreactivity was only measurable in extracts of the whole brain (Table IV-2), pituitary gland (Fig. IV-3) and myenteric plexus (Fig. IV-5), even after concentration of the extracts by freeze-drying (Fig. IV-4) or Sep-Pak separation (Fig. IV-6). The amount of IR PTH material in the concentrated brain extracts was sufficient to almost completely suppress tracer binding to the PTH antisera (Figs. IV-4 and 6), although the brain IR PTH concentration was consistently less than that present in the pituitary gland (Table IV-1). The IR PTH concentration in the brain was, nevertheless, consistently higher than that present in plasma (Table IV-1). The IR PTH concentration in the basomedial hypothalamus of the frog, turtle, chicken, and rat brain was, however, comparable with that found in the pituitary gland and much higher than that in the rest of the brain (Table IV-3). The IR PTH concentration in the guinea pig (2.3 ng/mg) and rat myenteric plexus (1.2 ng/mg) was lower than that in most brain and pituitary extracts, but higher than in plasma.

When extracts of the rat hypothalamus (Fig. IV-7) and pituitary gland (Fig. IV-8) were separated by HPLC, protein fractions, eluting in 38 to 42% acetonitrile had measurable IR PTH, in fractions that coeluted with hPTH-(1-84). Further purification of combined IR fractions resulted in a broadening of the IR peak (Fig. IV-8b).

The IR PTH concentration in the rat pituitary gland and in the rest of the brain was not affected by dietary calcium status, although the plasma IR PTH concentration was consistently elevated in

rats fed a low-calcium diet (Fig. IV-9) and the plasma ionized calcium level (1.29 ± 0.02 mM, n = 12), was reduced in comparison with rats maintained on normal (1.37 ± 0.02 mM, n = 12) or high-calcium (1.40 ± 0.02 mM, n = 12) diets.

DISCUSSION

These results clearly demonstrate the presence of PTH immunoreactivity in the brain, hypothalamus, and pituitary gland of amphibian, reptilian, avian, and mammalian species and the myenteric plexus of rat and guinea pig ileum. This immunoreactivity is a result of the presence of a heat-stable nondialyzable peptide and hence is similar in these regards to authentic PTH (Habener and Potts 1976). Although a purified single IR-PTH peak was not obtained, it was demonstrated that this immunoreactivity coelutes with PTH when separated by reverse-phase HPLC. By use of antisera directed against the (35-84) or (44-68) region of the intact molecule, PTH-like immunoreactivity has previously been found, in comparable concentrations, in the brain and pituitary glands of fish (Harvey *et al.*, 1987) and mammals (Balabanova *et al.*, 1985; Balabanova *et al.*, 1986b) even though these regions of the PTH molecule may not be tightly conserved. However, PTH-like immunoreactivity, measured by NH_2-terminal PTH antisera, has also been detected in the pituitary glands of fish (Harvey *et al.*, 1987) and was reported to be present in the pituitary glands of a number of mammalian species (Parsons *et al.*, 1978). Peptides closely resembling mammalian PTH would thus appear to occur

ubiquitously in vertebrate pituitary glands, and the results of the present study suggest that they may occur ubiquitously in brain also. These findings may thus have evolutionary significance, since parathyroid glands only appeared in the evolution of the vertebrates in the tetrapods, with the transition from an aquatic to terrestial environment. The presence of PTH-like peptides in the brain and pituitary gland of fish (Harvey *et al.*, 1987) and mud puppies (Fig. IV-3) might indicate that these tissues are ancestral sites of PTH production, since these species lack encapsulated parathyroid glands (Pang *et al.*, 1982). This possibility is supported by the finding of cell bodies in the preoptic region of several vertebrate brains that react immunocytochemically with the same PTH antisera (Pang *et al.*, 1988).

The presence of PTH immunoreactivity in the plasma of these species, at concentrations comparable with those in "higher" vertebrates, also suggests that the PTH-like material in the brain and pituitary gland is actively secreted. This possibility is supported by the demonstration that IR PTH is secreted from the pituitary glands of sheep *in vitro*, in response to media of low-calcium content and by dibutyryl cAMP (Balabanova *et al.*, 1986b). Although the content of IR PTH in the brain and pituitary gland was not increased in Ca-deficient rats (Fig. IV-6), the regulation of IR PTH secretion from the mammalian pituitary gland would appear to be very similar to the regulation of PTH secretion from the parathyroid gland (Habener and Potts 1976) and it is thus possible that extraparathyroidal PTH may also contribute to the

plasma IR PTH concentration in higher vertebrates. Whether the release of IR PTH in the brain and pituitary of lower vertebrates is regulated in the same manner remains to be demonstrated.

The widespread occurrence of IR PTH in brain and pituitary tissue suggests that it serves an important, although still mysterious, physiological function in the vertebrates. The IR PTH material in the brain and pituitary gland may play a role as a neurohormone in calcium regulation, but it is also possible that it may act as a neurotransmitter or neuromodulator within the nervous system. Recently Pang *et al.* (1986) observed that PTH has many actions in addition to its role in plasma calcium and phosphate regulation and these actions parallel those of β-adrenergic stimulation. It was suggested that PTH may be released as a neurotransmitter and act on specific PTH receptors, since propranolol (a β-adrenergic antagonist) had no inhibitory affect on these PTH actions. The demonstration of IR PTH in neural tissue especially in the intestinal myenteric plexus, is consistent with this hypothesis. A similar role in neural transmission has also been suggested for the calcitonin-like material found in neural tissue (Flynn *et al.*, 1981; Twery *et al.*, 1986a; Twery *et al.*, 1986b). Moreover, as the IR calcitonin found in neural tissue is now known to be a result of the presence of a calcitonin gene-related peptide (Tschopp *et al.*, 1984; Zaidi *et al.*, 1986), it is possible that the IR PTH in the brain may also be a product of a PTH gene-related peptide.

Table IV-1. Immunoreactive parathyroid hormone in boiled dialyzed extracts of vertebrate tissues.

Speices	Plasma, ng PTH-(1-84)/ml				Brain, ng PTH-(1-84)/g wet wt				Pituitary, ng PTH-(1-84)/g wet wt		
	Anti-PTH-(53-64)	n	Anti-PTH-(48-53)	n	Anti-PTH-(53-64)	n	Anti-PTH-(48-53)	n	Anti-PTH-(53-64)	n	AntiPTH-(48-53)
Mammals											
Rabbit	0.48±0.04	4	0.23±0.01	4	3.66±0.47	4	5.12±0.68	4	9.1±2.0	4	12.46±4.1
Rat	0.65±0.07	33	0.56±0.03	47	3.40±0.46	4	5.47±0.38	4	14.7±2.2	4	18.39±3.7
Hamster	0.43±0.04	4	0.23±0.12	4	3.44±0.46	4	7.32±0.84	4	ND		ND
Mouse	0.38±0.02	6	0.20±0.01	6	3.47±0.38	6	8.31±0.74	6	ND	ND	
Birds											
Quail	0.39±0.07	21	ND		1.24±0.28	15	ND		12.5±3.6	14	ND
Chicken	0.51±0.05	4	0.22±0.01	4	2.07±0.76	4	9.9±0.2	3	13.3±1.2	15	ND
Duck	0.52±0.09	4	0.19±0.02	4	3.06±0.24	4	7.7±1.1	4	ND		ND
Reptiles											
Turtles	0.38±0.06	4	0.33±0.01	4	7.64±0.93	4	11.3±1.8	4	10.1±0.7	4	ND
Garter Snake	0.21±0.03	3	0.28±0.02	4	4.26±0.34	4	4.50±0.3	8	ND		ND
Amphibians											
Toads	0.39±0.02	6	0.21±0.03	8	5.09±1.13	4	ND		3.61±3.0	6	ND
Bullfrog	0.59±0.04	6	0.39±0.09	4	4.49±0.66	14	ND		20.1±3.6	15	ND
Mud puppy	0.35±0.04	6	0.28±0.12	6	ND		ND		ND		ND

Values are means ± SE; n, no. of animals. Immunoreactive parathyroid hormone was not determined in muscle, liver, or kidney tissue extracts.
ND, Not determined.

Table IV-2. Apparent parathyroid hormone-like immunoreactivity in vertebrate tissues.

Tissue Extract	IR PTH-(1-84), ng/g wet wt		
	Before boiling	After boiling	After boiling and dialysis
Rat			
Brain	17.35	4.64	4.23
Muscle	6.38	ND	ND
Liver	10.95	1.53	ND
Kidney	15.96	1.88	ND
Quail			
Brain	20.05	8.31	5.69
Muscle	6.84	1.89	ND
Liver	9.88	1.59	ND
Kidney	12.25	2.96	ND
Turtle			
Brain	19.45	5.19	2.88
Muscle	5.16	ND	ND
Liver	10.74	ND	ND
Kidney	16.17	2.13	ND
Bullfrog			
Brain	19.40	8.21	5.47
Muscle	11.69	ND	ND
Liver	30.23	ND	ND
Kidney	24.30	1.64	ND

IR PTH, immunoreactive parathyroid hormone levels as determined by cross-reactivity with PTH-(48-64), (53-64) antisera; ND, not detectable.

Table IV-3. Immunoreactive parathyroid hormone in vertebrate tissues.

Tissue	IR PTH-(1-84), ng/mg protein	
	Method A	Method B
Bullfrog		
Brain	0.45	0.87
Hypothalamus	5.87	4.36
Pituitary	6.45	5.52
Turtle		
Brain	0.32	0.36
Hypothalamus	3.81	3.52
Pituitary	3.68	3.89
Chicken		
Brain	0.53	0.49
Hypothalamus	2.99	3.16
Pituitary	3.46	3.68
Rat		
Brain	0.49	0.47
Hypothalamus	3.98	3.82
Pituitary	4.01	4.06

IR PTH-(1-84), immunoreactive parathyroid hormone; method A, IR PTH-(1-84) level determined using PTH-(48-64), (55-64) antisera; method B, IR PTH-(1-84) level determined using PTH-(48-64), (48-53) antisera.

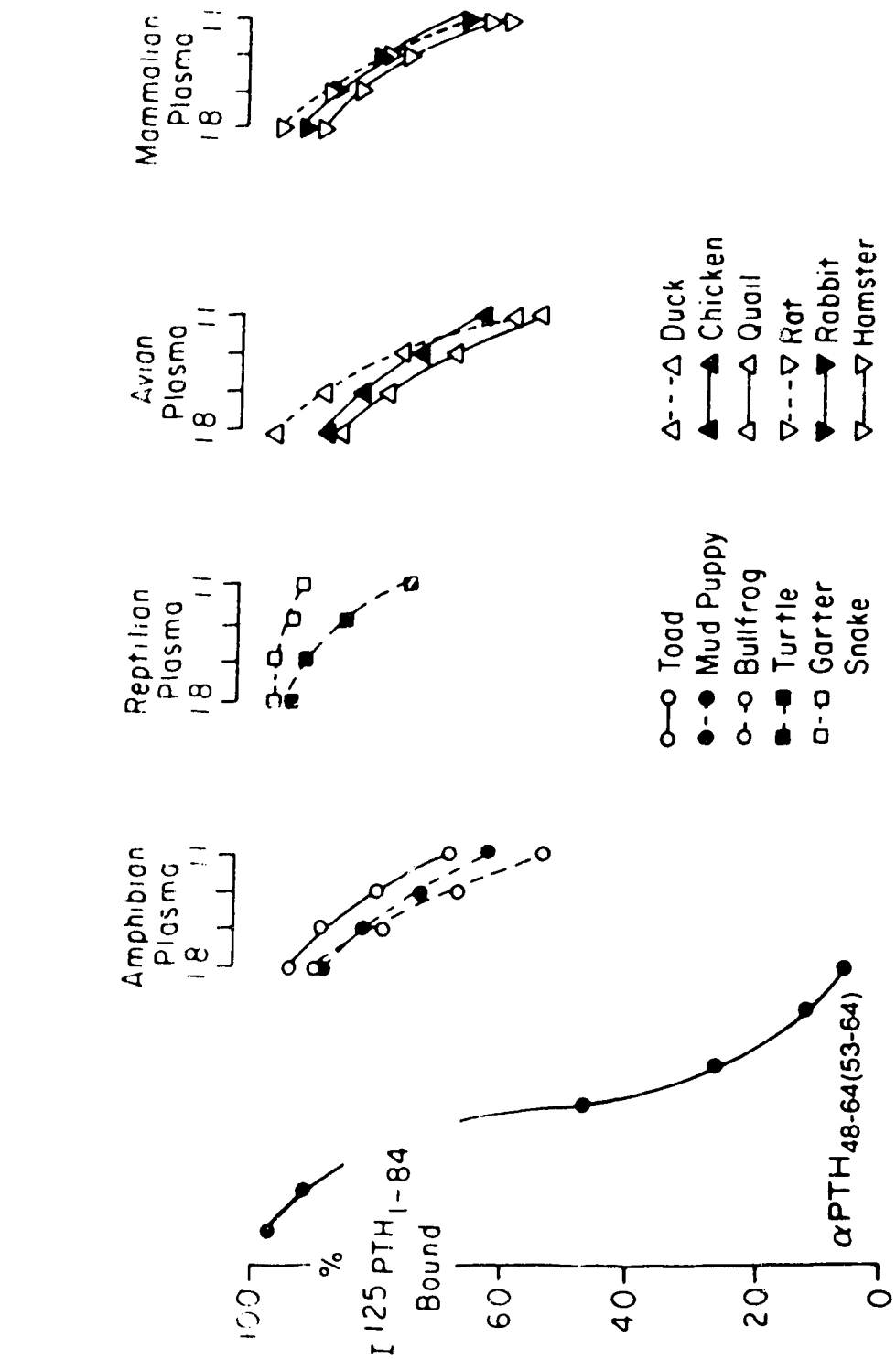


Figure IV-1 Cross-reaction of plasma from various vertebrate species with antisera directed against the immunoreactive parathyroid hormone PTH-(48-64), (53-64).

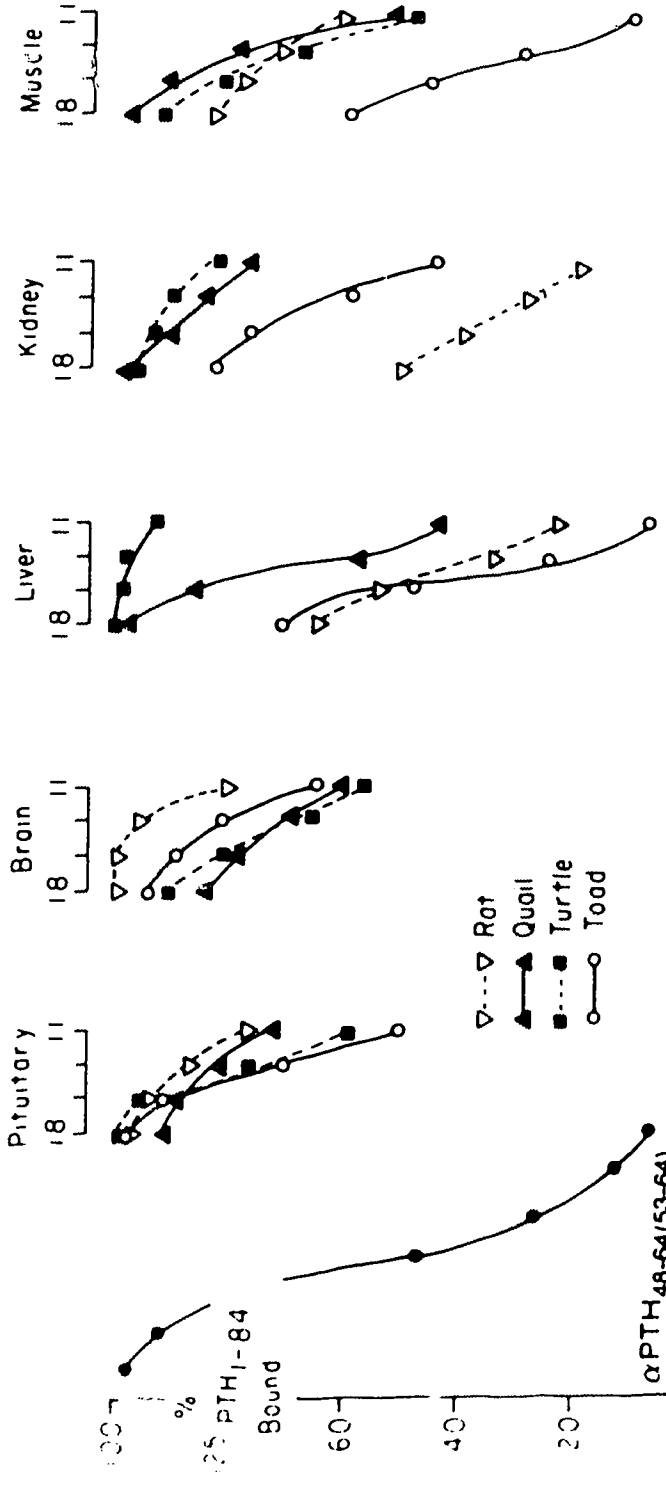


Figure IV-2 Cross-reaction of crude saline homogenates of pituitary, brain, liver, kidney, or muscle tissue. from rat, quail, turtle, and toad with PTH-(48-64), (53-64) antisera.

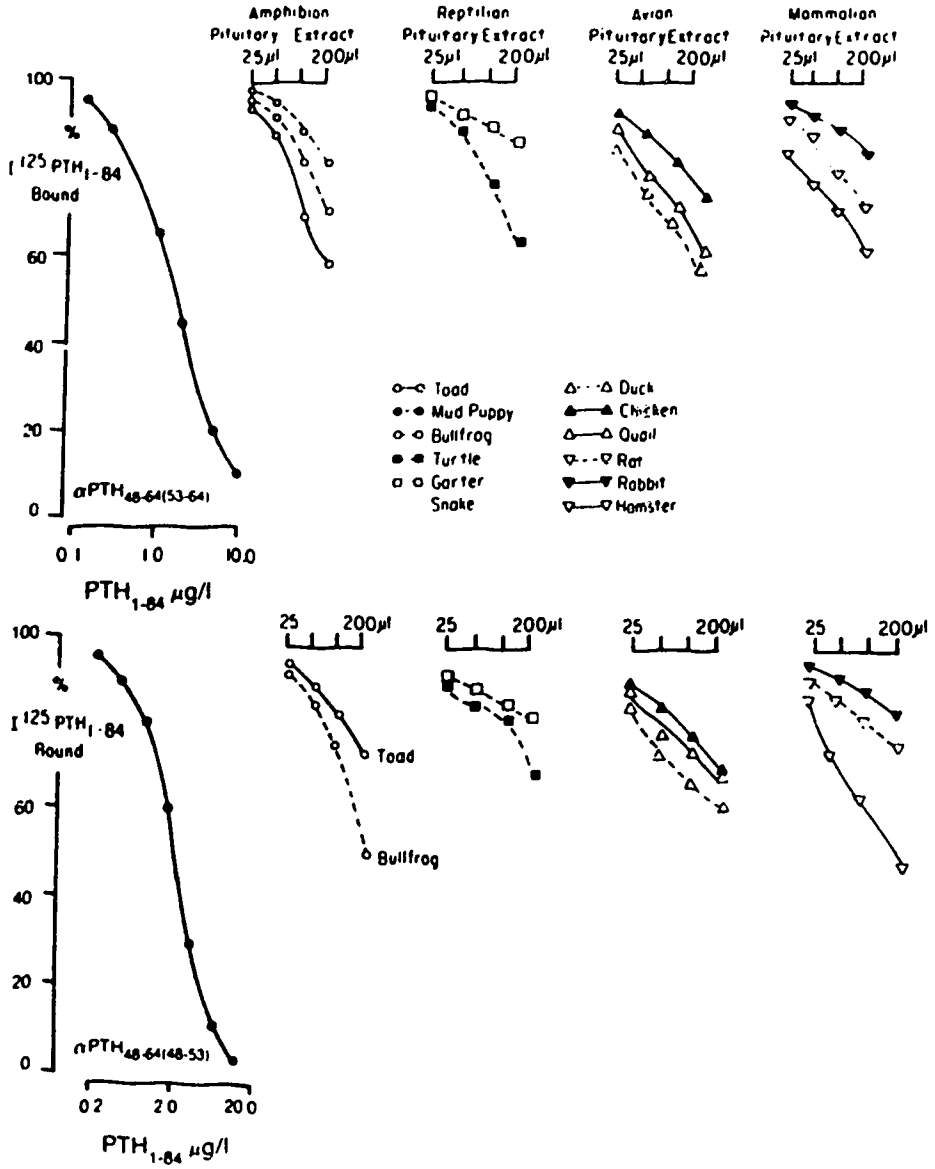


Figure IV-3. Cross-reaction of boiled dialyzed extracts of amphibian, reptilian, avian, and mammalian pituitary glands with PTH-(48-64), (53-64) or PTH-(48-64), (48-53) antisera.

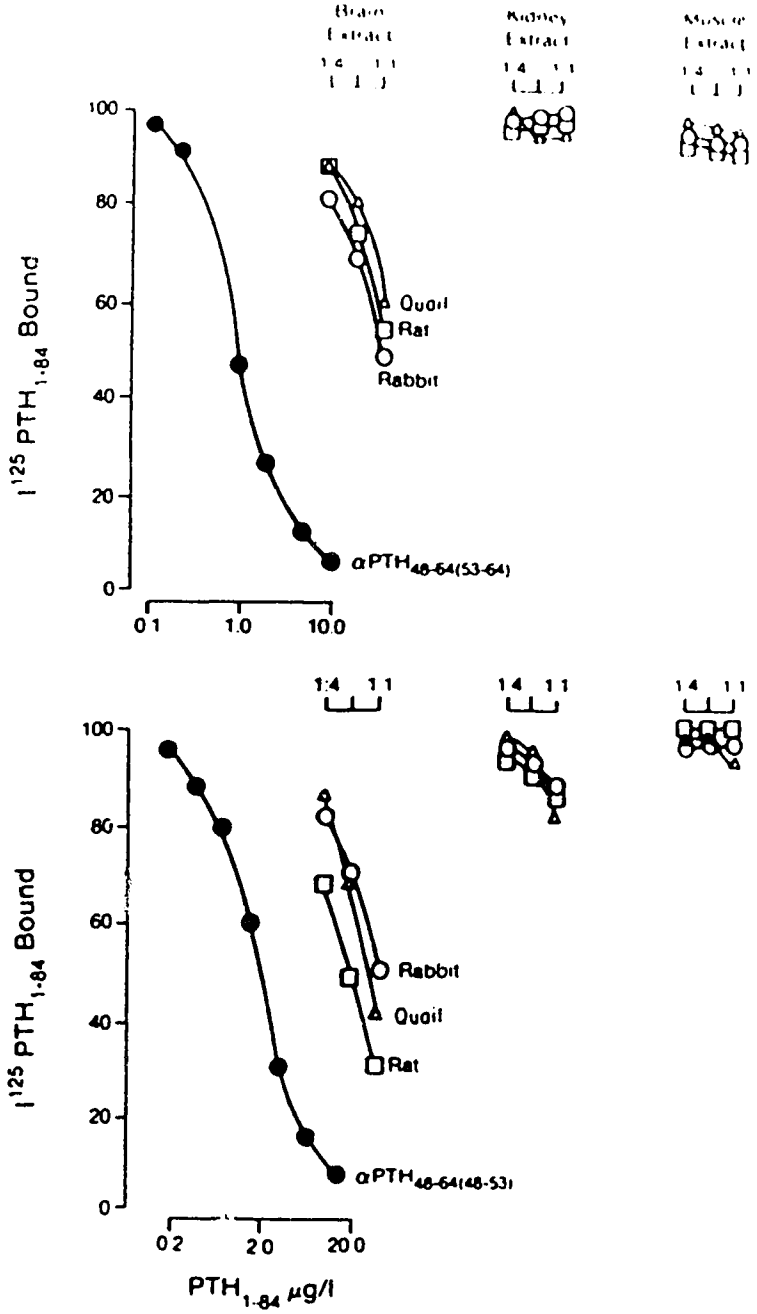


Figure IV-4. Cross-reaction of boiled, dialyzed extracts of rat, rabbit, and quail brain (g/l) with PTH-(48-64), (53-64) and PTH-(48-64), (48-53) antisera.

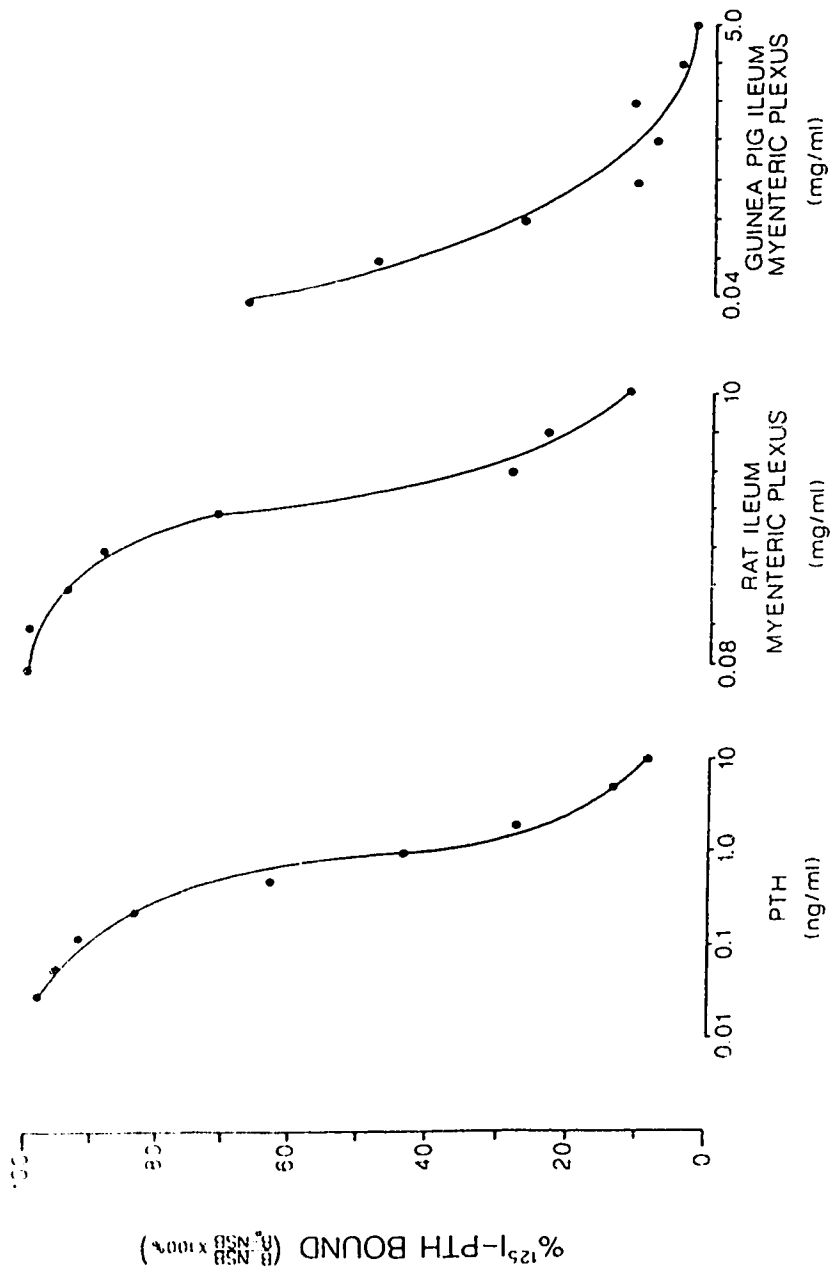


Figure IV-5 Cross-reaction of boiled dialyzed extracts of rat and guinea pig myenteric plexus with PTH-(48-64) antisera.

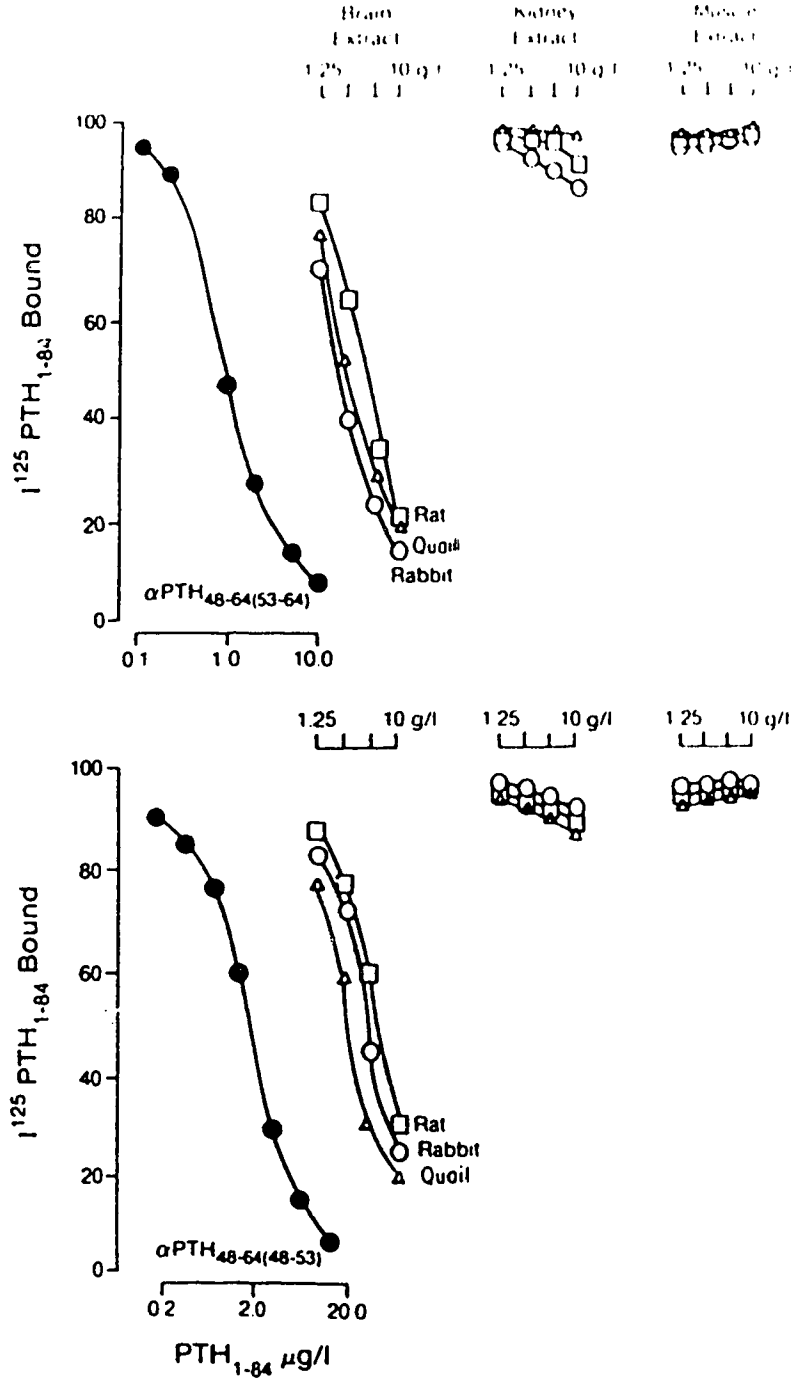


Figure IV-6. Cross-reaction of boiled, dialyzed Sep-Pak C_{18} purified extracts of rat, rabbit, and quail brain with PTH-(48-64), (53-64) and PTH-(48-64), (48-53) antisera.

Figure IV-7. Elution profile of rat pituitary extract separated by reverse-phase high-performance liquid chromatography in a 0-80% acetonitrile gradient in comparison with human PTH-(1-84) and IR PTH content of protein fractions.

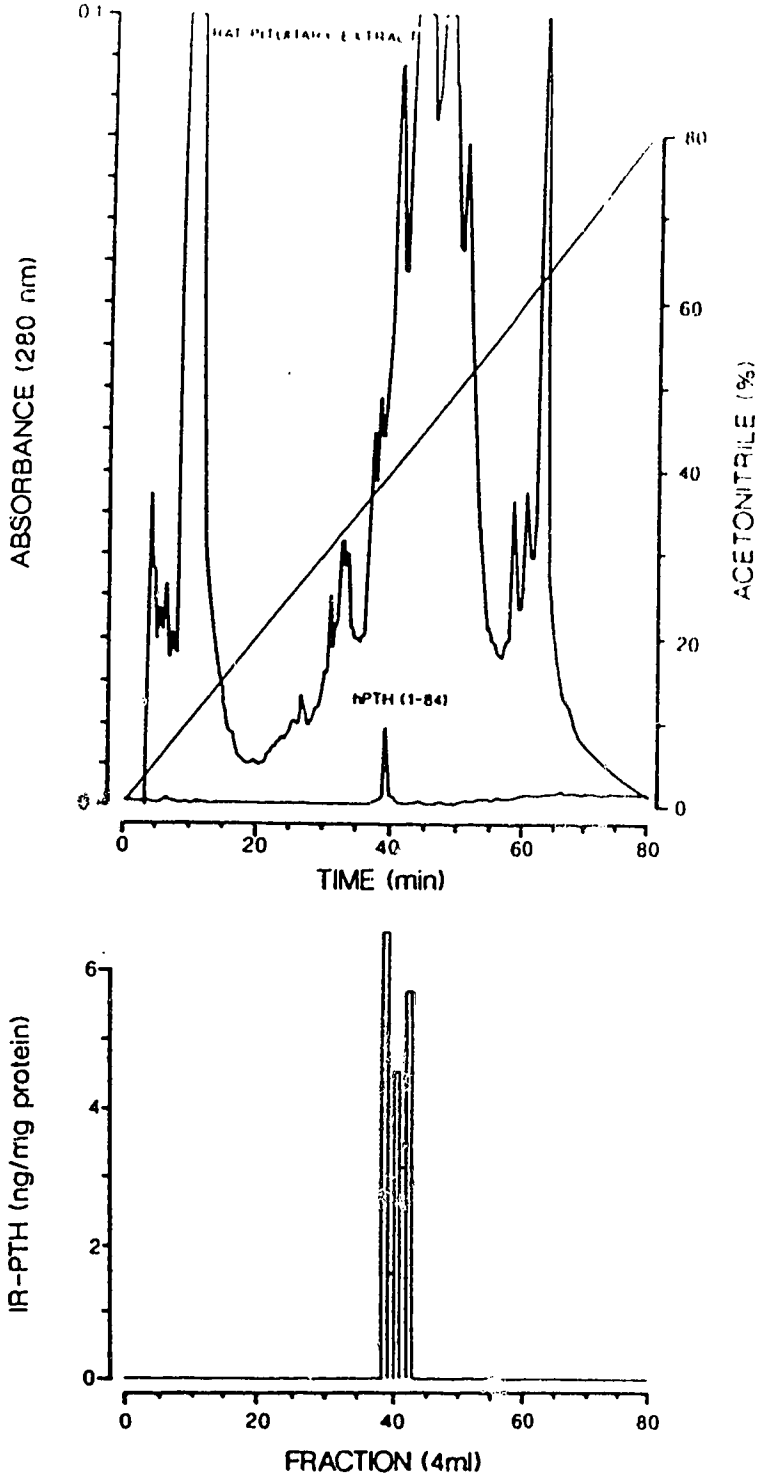
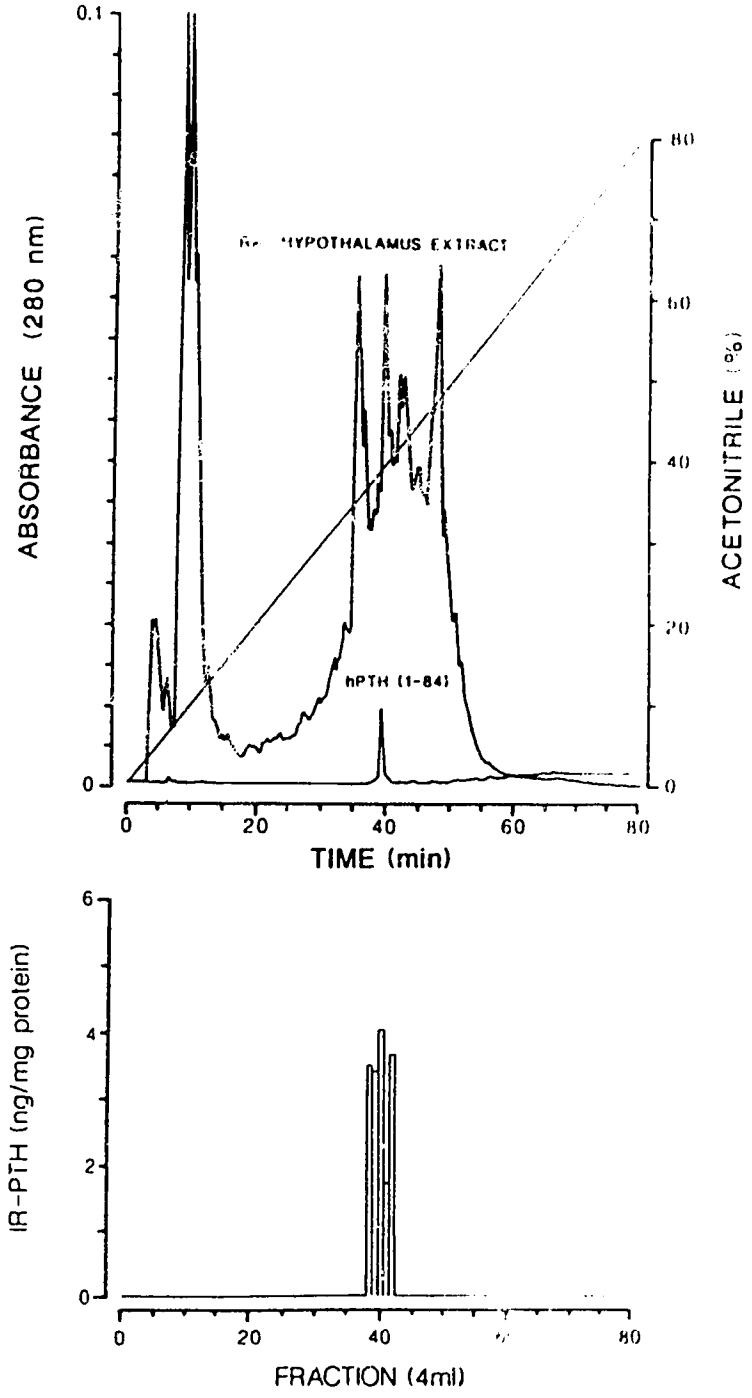
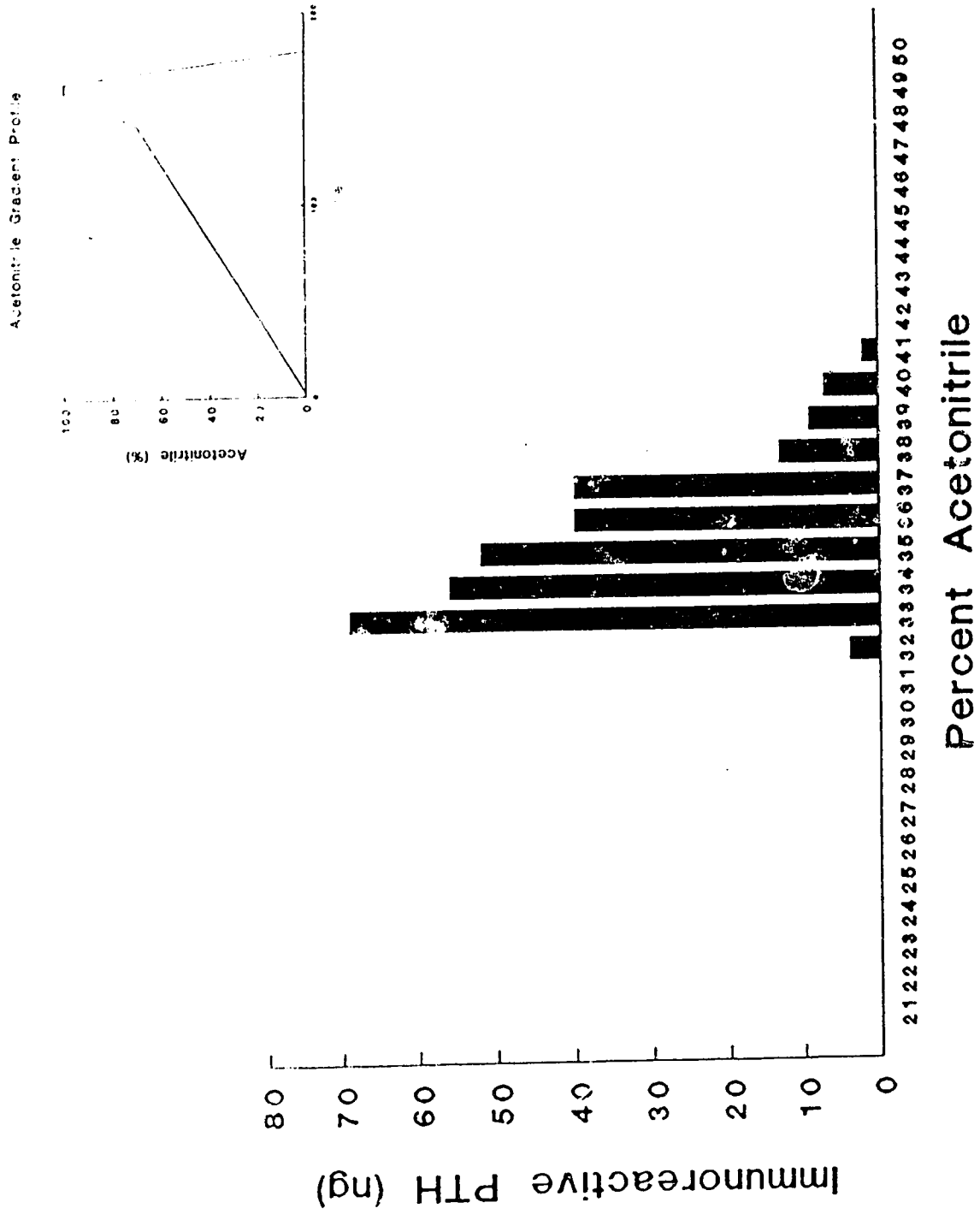

ABSORBANCE (280 nm)
ACETONITRILE (%)
hPTH (1-84)
TIME (min)
IR-PTH (ng/mg protein)
FRACTION (4ml)

Figure IV-8. a) Elution profile of rat hypothalamic extract separated by reverse-phase high-performance liquid chromatography in a 0-80% acetonitrile gradient in comparison with human PTH-(1-84) and IR PTH content of protein fractions. b) Elution profile of combined IR fractions, chromatographed in a reduced acetonitrile gradient slope.


0.1
0
ABSORBANCE (280 nm)
HYPOTHALAMUS EXTRACT
hPTH (1-84)
80
60
40
20
0
ACETONITRILE (%)
0
20
40
60
80
TIME (min)
6
4
2
0
IR-PTH (ng/mg protein)
0
20
40
80
FRACTION (4ml)


Immunoreactive PTH (ng)
0
10
20
30
40
50
60
70
80
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
Percent Acetonitrile
Acetonitrile (%)
0
20
40
60
80

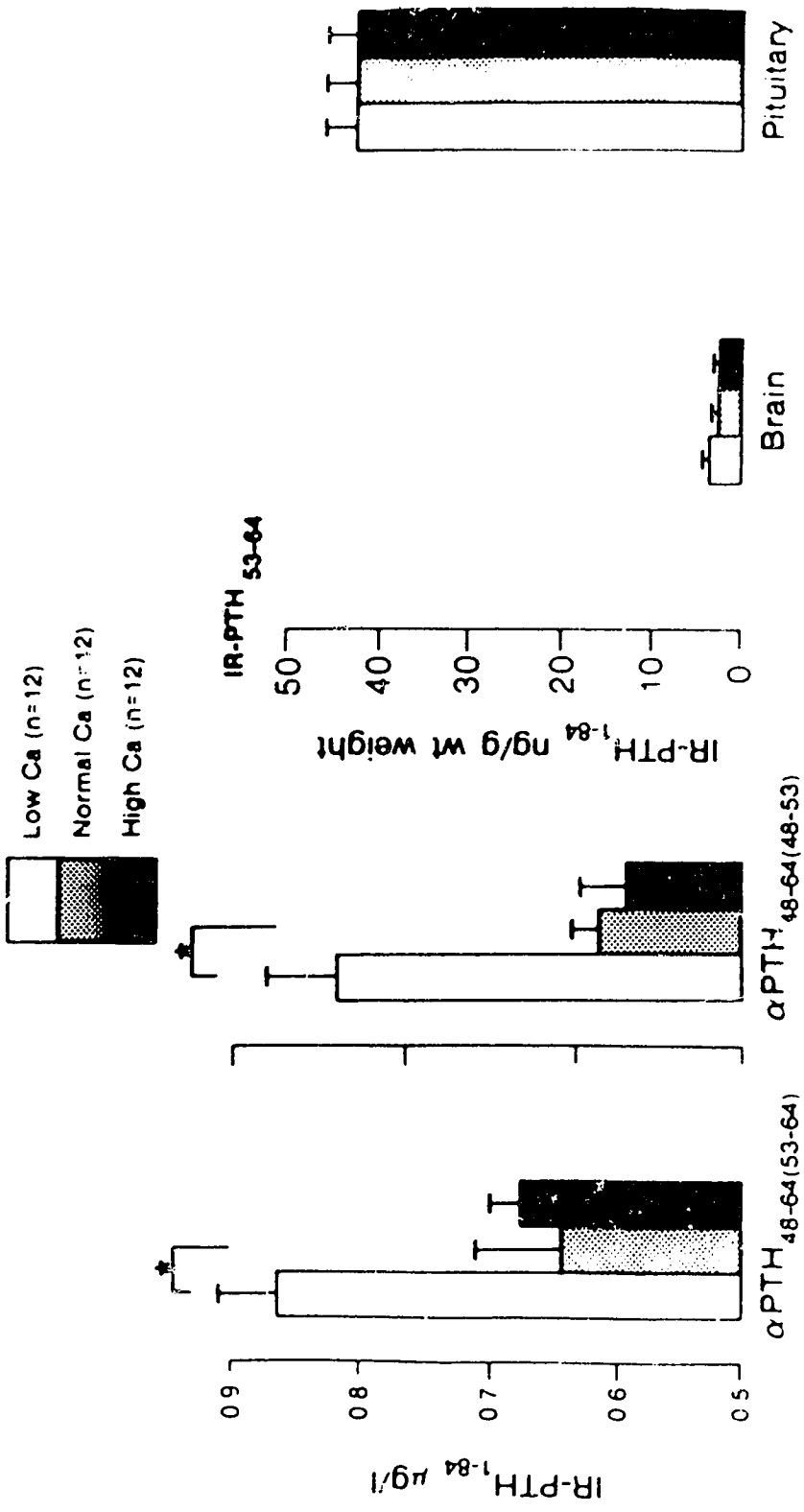


Figure IV-9 Concentration of immunoreactive (IR) parathyroid hormone (PTH) in plasma, brain, and pituitary glands of rats fed for 10 days of diet with a low-(0.0%) normal-(0.5%), or high-(2-5%) calcium content. Statistical differences are indicated (*). All values are means ± SE (n = 12).

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CHAPTER V

PARATHYROID HORMONE MESSENGER RIBONUCLEIC ACID IN THE RAT HYPOTHALAMUS[1]

1. A version of this chapter is in press. Fraser RA, Kronenberg HM, Pang PKT, Harvey S, 1990 Parathyroid hormone messenger ribonucleic acid in the rat hypothalamus. Endocrinology.

INTRODUCTION

Ectopic hormone production has been demonstrated by the diverse distribution of immunoreactive (IR) polypeptide hormones in endocrine and non-endocrine tissues (Pearse, 1981; Krieger, 1984; Kastin *et al.*, 1987; Said, 1987). Parathyroid hormone (PTH), the major peptide regulator of blood calcium is synthesized primarily in the parathyroid gland. IR PTH however has also been found in human cerebrospinal fluid (Balabanova *et al.*, 1984) and in the brain and pituitary gland of sheep (Balabanova *et al.*, 1985; 1986). More recently, heat-stable, non-dialysable IR PTH-like activity has been localized in the brain and pituitary gland of piscine (Harvey *et al.*, 1987), amphibian, reptilian, avian and mammalian species (Pang *et al.*, 1988a; 1988b). This IR PTH has been shown to co-elute with authentic PTH from reverse-phase Sep Pak C_{18} preparative and high performance liquid chromatography (HPLC) columns (Pang *et al.*, 1988a). Immunocytochemical (ICC) studies have also indicated that peptides with IR PTH are confined to perikarya in specific hypothalamic nuclei (Pang *et al.*, 1988b). The possibility that the PTH gene may, therefore, be expressed in the brain has been determined in the present study, using a specific complementary ribonucleic acid (RNA) probe to PTH mRNA.

MATERIALS AND METHODS

RNA Preparation

Total RNA was prepared following a modified procedure of Okayama *et al.* (1987). Briefly, hypothalami, livers and parathyroid

glands were dissected from 250 g male Sprague-Dawley rats (Harlen Co., IN, USA) and immediately frozen in liquid nitrogen. Approximately 1-2 g of the liver, total hypothalami and 200 μg of the parathyroidal frozen tissue, each in 5.5 M guanidinium thiocyanate solution (1:10 w/v), were homogenized by a polytron (Brinkman Instruments, IL, USA) and centrifuged at 1500 x g at 22°C for 5 min. The supernatants were passed through an 18 guage needle to sheer DNA (Maniatis *et al.*, 1982), and centrifuged at 5,000 x g at 15°C for 20 min. The supernatants were then subjected to isopicnic ultracentrifugation (±25,000 x g at 15°C for 24 h) through a cesium trifluoroacetic acid (CsTFA) bed (density 1.51 ± 0.02 g/ml; Pharmacia Fine Chemicals, Uppsala, Sweden), containing 0.1 M EDTA, pH 7.0. The RNA pellets were resuspended in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA buffer (TE) (Maniatis *et al.*, 1982), heated to 65°C and centrifuged to remove insoluble material. The yield of RNA was 0.094 ± 0.01% of total tissue weight, as determined by spectral analysis at 260 nm and its purity was assessed by ethidium bromide staining after 1% agarose minigel electrophoresis (Maniatis *et al.*, 1982).

Polyadenylated (poly A^+) RNA was separated from total hypothalamic RNA using oligo-deoxythymidine (oligo-dT) cellulose spin columns (Pharmacia Fine Chemicals, Uppsala, Sweden), and precipitated with 10 M ammonium acetate (0.2 vol/vol) and ethanol (3 vol/vol) at -80°C for 1 h. The poly A^+ RNA pellet was dried and resuspended in diethylpyrocarbonate (DEPC)-treated water prior to spectral quantification and minigel analysis.

Ribonucleic Acid Probe Synthesis

A portion of the rPTH gene (Hienrich *et al.*, 1984) containing most of exon III and intron B was subcloned into a pGEM4 vector (Promega Corporation, Madison, WI, USA) by Dr. Gerhard Heinrich (Fig. V-1a). A second plasmid (Fig. V-1b) was constructed by inserting the *Bam* H1/*Hind* III chicken PTH (cPTH) (Khosla *et al.*, 1989) fragment into pGEM2 vector (Promega Corporation, Madison, WI, USA). The complementary, or antisense, sequence of rPTH mRNA and the sense sequence of cPTH mRNA, were transcribed *in vitro* using *Hind* III-digested (Boehringer Mannheim, Dorval, Quebec, Canada) rPTHGEM4 or cPTHGEM2 as templates and SP6 polymerase (Bethesda Research Laboratories, Bethesda, MD, USA), in the presence of 25 μCi [^{32}P]CTP (3,000 Ci/mmol) for probing Northern blots and [^{35}S]CTP (800 Ci/mmol) (New England Nuclear Missassauga, Ontario, Canada) for probing *in situ* hybridization, following a modified procedure of Melton *et al.* (1984). The sense sequence of cPTH mRNA was used to construct a non-specific probe since it was of similar size (430 bp) as the rPTH gene fragment (375 bp) and could be similarly synthesised. rPTH mRNA was not used to construct a sense riboprobe in view of the possibility that small amounts of the antisense sequence could be simultaneously transcribed *in vivo* (Kinelman and Kirschner, 1989). The probes were purified from unincorporated ribonucleic acids by three consecutive 10 M ammonium acetate (0.2 vol/vol), isopropanol (3 vol/vol) precipitations and resuspended in DEPC-treated water.

Northern Blot Analysis

Total RNA from rat parathyroid gland (1 μg) and poly A^+ RNA from rat hypothalamus (10 μg), in 50% formamide, 0.1% formaldehyde and 1X MOPS (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) (Maniatis *et al.*, 1982) were electrophoresed through a 1.2% agarose and 3.1% formaldehyde gel containing 1X MOPS. The RNA was transferred by capillarity to nitrocellulose, which was then rinsed in 6X SSC (1X SSC = 0.15 M NaCl 0.015 M sodium citrate, pH 7.2) and baked at 80°C for 2 h under a vacuum.

The Northern blots were prehybridized for at least 2 h at 65°C in 50% formamide containing 5X PIPES (0.75 M NaCl, 25 mM PIPES, 25 mM EDTA, pH 6.8), 5X Denhardt's (0.1% Ficoll, 0.1% BSA, 0.2% SDS and 0.1% polyvinylpyrrolodine), salmon sperm DNA (100 μg/ml) and yeast tRNA (100 μg/ml) (Sigma Chemical Co., St. Louis, MO, USA) and then hybridized under the same conditions, for 12 h in the presence of the newly synthesized RNA probes. The blots were then serially washed at room temperature and twice at 68°C in 0.2% SDS, containing 2, 0.5 and 0.05X SSC, respectively, prior to exposure to x-ray film (X-OMAT AR, Kodak, Rochester, NY, USA) for periods of 2 h to 1 week.

Polymerase Chain Reaction (PCR)

Rat parathyroid gland total and hypothalamic poly A^+ RNA was reversed transcribed by ribonuclease H^- Moloney murine leukemia virus (M-MLV) reverse transcriptase (100 units, Bethesda Research Laboratories, Gaithersburg, MD, USA) in the presence of 3'-oligomer

rPTH antisense primer (50 pmol, Fig. V-2b), deoxynucleotides (1.25 mM of each, Boehringer Mannhein, Dorval, Quebec, Canada) and 1X PCR buffer (50 mM KCl, 10 mM Tris HCl pH 8.4, 1.5 mM $MgCl_2$ and 20 μg/ml gelatin) (Kawasaki, 1990). The reactions were diluted with DEPC-treated water (500:1, vol:vol) and an aliquot of each (1/1000 of total vol) was used in a PCR (Kawasaki, 1990) mixture containing both 5'-oligomer rPTH sense and 3'-oligomer rPTH antisense primers (15 pmol of each, Fig. V-2), deoxynucleotides (1.25 mM of each), 1X PCR reaction buffer and *Thermus aquaticus* (*Taq*) DNA polymerase (5 units, Boehringer Mannheim, Dorval, Quebec, Canada). The mixture was overlayed with mineral oil (v:v), heated to 94°C for 2 min prior to 30 cycles of 65°C annealing for 1 min, 72°C extension for 30 sec and 94°C denaturing for 30 sec in a thermal reactor (Tyler Instruments, Edmonton, Alberta, Canada). Rat hypothalamic PCR reaction product (0.04 vol) was reamplified under the identical conditions. As a negative control, liver poly A^+ RNA was similarly reversed transcribed and subjected to PCR, as described above.

In Situ Hybridization

Sodium pentabarbitol-anesthetized Sprague-Dawley rats were perfused with phosphate-buffered saline (PBS) containing EGTA (100 μg/l) and then with 4% paraformaldehyde in phosphate buffer (pH 7.0). Whole brains were dissected and post-fixed in 4% paraformaldehyde at 4°C overnight and cryoprotected by sequential saturation in 10, 15 and 20% sucrose-phosphate buffer. Coronal sections, 10 μm, were cut using a cryostat (Reichert-Jung,

Cambridge Ins. CombH, Heidelberg, West Germany) and mounted onto gelatin (0.4%) chromium potassium sulfate (0.04%)-coated slides.

Tissue sections were fixed with 4% formaldehyde in PBS, perforated with 50 mM Tris HCl (pH 7.6) and 5 mM EDTA containing proteinase K (20 μg/ml, Boehringer Mannheim, Dorval, Quebec, Canada) and dried with ethanol, prior to prehybridization in hybridization buffer (50% formamide, 5X PIPES, 5X Denhardt's, 0.2% SDS, 100 mM dithiothreitol and 250 μg/ml of salmon sperm DNA and yeast tRNA) in a humidified chamber at 42°C for 2 h (Miller *et al.*, 1989). Alternate sections were hybridized at 42°C for 12 h in hybridization buffer containing either rPTH antisense or cPTH sense RNA probes. Slides were then serially washed at room temperature (21^0C) in 4X SSC, initially in the presence of 10 mM β-mercaptoethanol. The non-hybridized RNA probes were then digested at 37°C with 50 μg/ml ribonuclease A (Boehringer Mannheim, Dorval, Quebec, Canada), in 0.5 M NaCl in TE, followed by a 2X SSC wash at room temperature and finally a 0.1X SSC wash at 42°C. Air-dried slides were dipped in autoradiographic emulsion (NTP-2, Kodak, Rochester, NY, USA) and exposed 14 d before developing.

RESULTS

Northern Blot Analysis

As expected, potent hybridization of the rPTH antisense riboprobe with RNA extracted from the parathyroid gland was observed within 2 h of exposure (lane 1, Fig. V-3). After 7 days, weak hybridization with hypothalamic poly A^+ RNA was observed with a moiety that

co-migrated with the signal detected in the parathyroid gland (lane 2, Fig. V-3). Under the same conditions and using equivalent amounts of the riboprobe, specific hybridization with liver RNA could not be detected (data not shown), although non-specific hybridization with the 28S and 18S band (located on the ethidium bromide-stained gel; data not shown) was evident. Non-specific hybridization with these bands was also indicated with the extract of hypothalamic poly A^+ RNA. Identical results were also demonstrated using three further poly A^+ RNA preparations (data not shown).

Polymerase Chain Reaction (PCR)

The PCR conducted with parathyroid gland cDNA produced a single intense band smaller than the 434 bp marker and larger than the 298 bp marker (lane 1, Fig. V-4) as viewed on the ethidium bromide stained gel (lane 2 Fig. V-4). Reamplification of rat hypothalamic cDNA also produced a band of equal size to that of rat parathyroid gland (lane 4, Fig. V-4). Amplification of liver cDNA did not reveal a PCR product (lane 3, Fig. V-4).

In Situ Hybridization

Exposure of the rat brain sections to the emulsion for 14 d indicated specific bilateral hybridization with rPTH riboprobe in the supraoptic (SO) nuclei (Figs. VI- 5a, -5b and -6). Specific hybridization was also demonstrated in the paraventricular lateral magnocellular (PaLM) nuclei (Figs. VI-5d, -5e and -6). No hybridization with an equivalent amount of the non-specific cPTH riboprobe was observed in adjacent sections of these or other

hypothalamic nuclei (Figs. VI-5c and 5f). Specific hybridization could not clearly be detected in sections exposed to the emulsion for <7d, further indicating the low abundance of the message.

Under higher magnification, clustering of the rPTH signal was found in specific cells of the SO (Fig. V-5b). The tight clustering seen in the SO was also evident in the PaLM, but not the paraventricular nucleus (PaV) or periventricular nucleus (Pe), although the signal in these nuclei was greater than background (Fig. V-5c).

DISCUSSION

These results demonstrate, for the first time, that a PTH-like mRNA capable of hybridizing with rPTH antisense riboprobe is present in the hypothalamus of rats. Northern blot analysis indicates that this mRNA co-migrates with rPTH mRNA (Fig. V-3), and the absence of other hybridizing bands (except those due to 28S and 18S rRNA) demonstrates the specificity of this signal. Although the signal on the Northern is weak, it was readily detected (under the conditions used) by the increased sensitivity of *in situ* hybridization (Haase *et al.*, 1985). Moreover, amplification of rat hypothalamic cDNA by PCR produced a single band of equal size to parathyroidal PTH cDNA (383 base pairs; Heinrich *et al.*, 1984), intense enough to be viewed by ethidium bromide staining (Fig. V-4). Since the rat hypothalamic cDNA was reverse transcribed from cytoplasmic poly A^+

RNA, the PTH gene would therefore appear to be expressed in the rat hypothalamus, even though the message is of low abundance.

In view of the unique nucleotide sequence of PTH mRNA at the 3' terminus (Heinrich *et al.*, 1984) it is highly unlikely that mRNA other than PTH mRNA could hybridize with the riboprobe, given the stringency of the hybridization conditions used. Although Weir *et al.* (1990) reported that a PTH-related peptide (PTHrp) was expressed in rat brain, the sequence homology between PTH and PTHrp is restricted to a short sequence near the 5' end (Suva *et al.*, 1987). Since the RNA PTH probe, synthesized from rPTHGEM4, and the 5' primer oligonucleotide synthesized do not complement PTHrp mRNA, it is highly unlikely that the mRNA we detected was an expression of PTHrp gene. Furthermore, while PTH mRNA was detected in the hypothalamus, Weir *et al.* (1990) reported the expression of the PTHrp gene in extrahypothalamic tissues, particularly the cerebral cortex, and cerebral hemispheres. The possibility that the PTH gene may also be expressed in extrahypothalamic brain regions has yet to be examined, although Balabanova *et al.* (1986) detected IR-PTH throughout the brain.

In the present study, cells hybridizing with the PTH mRNA riboprobe were located within the SO and PaLM nuclei of the hypothalamus (Figs. V-5 and V-6). In contrast, however, PTH IR was determined by immunocytochemistry primarily within the PaV nuclei and to a lesser extent in the suprachiasmatic, Pe and SO nuclei of the mouse brain (Pang *et al.*, 1988b). PTH-like immunoreactivity has, nevertheless, also been located in the SO nuclei of other vertebrate

species (Kaneko and Pang, 1987; Pang *et al.*, 1988b). In view of this finding it is therefore probable that the IR peptides previously detected in the vertebrate brain resulted from the translation of PTH mRNA expressed in these nuclei. The synthesis or release of these peptides may, however, differ from that in the parathyroid gland, since the content of IR PTH in the rat brain was recently shown to be independent of hyper- and hypocalcemia (Pang *et al.*, 1988a), even though calcium depletion and vitamin D stimulated the release of IR PTH from sheep brain explants *in vitro* (Balabanova *et al.*, 1986). The role if any, for brain PTH in peripheral calcium homeostasis or in central function is also uncertain.

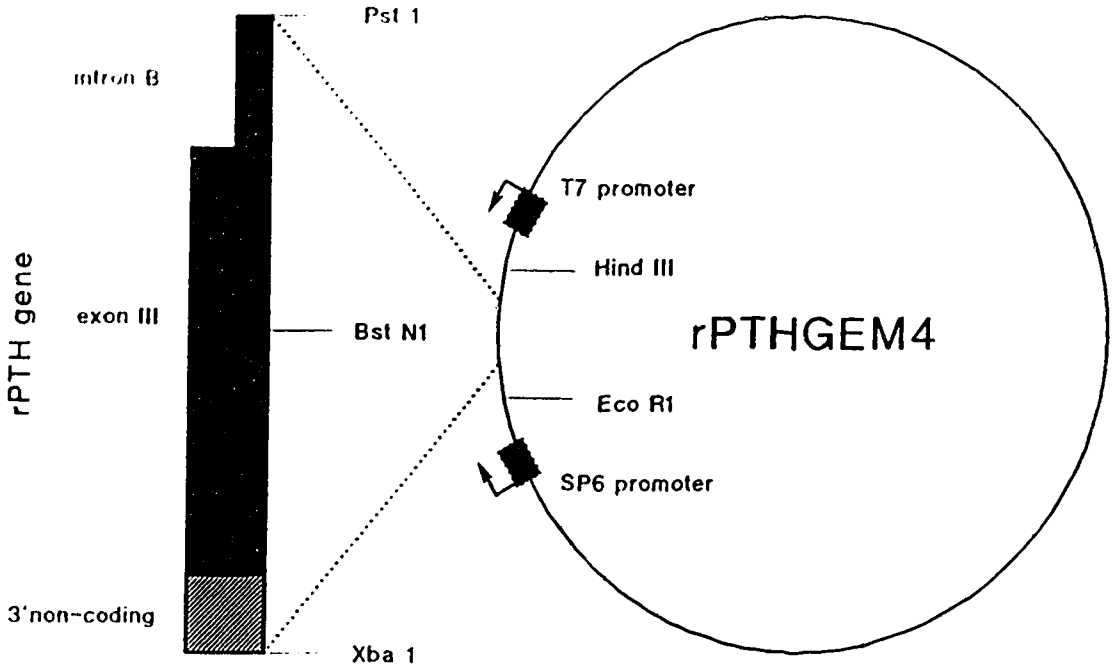
The locations of IR PTH and PTH mRNA within the brain are in hypophysiotropic regions of the hypothalamus that may regulate anterior and/or posterior pituitary function. The possibility that brain PTH may function as a hypophysiotropic releasing factor is suggested, since peptidergic IR PTH fibres terminate around hypophysial blood vessels in the external zone of the mouse median eminence of mammals and in the adenohypophysis of teleosts lacking portal blood vessels (Kaneko and Pang, 1987; Pang *et al.*, 1988b). The demonstration of increased prolactin secretion in mammalian species systemically injected with PTH or parathyroid gland extracts (Issac *et al.*, 1978; Castro *et al.*, 1980; Brickman *et al.*, 1981; Kruse *et al.*, 1981; Raymond *et al.*, 1982) may also indicate a neuroendocrine role of this peptide.

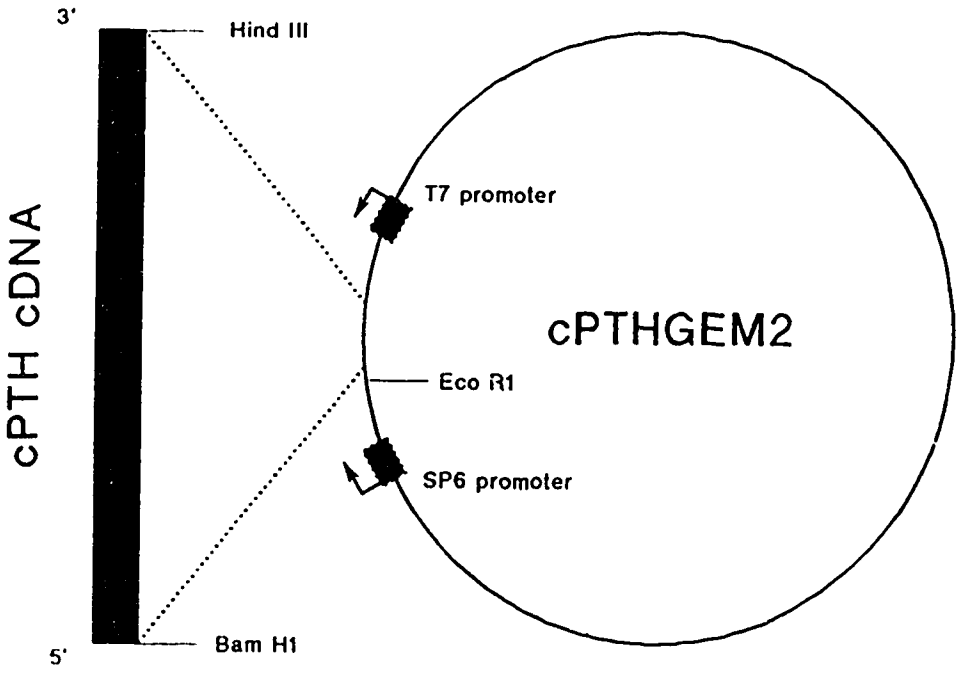
In view of the location of PTH within the brain, it is also possible that PTH may participate in neurotransmission. This

possibility is supported by the finding that PTH has peripheral actions on target tissues that parallel those induced by β-adrenergic stimulation (Pang *et al.*, 1986). Actions of PTH on neural tissue (inhibition of Ca^{++} channels in neuroblastoma cells) have also been demonstrated (Pang *et al.*, 1990) and PTH has also recently been shown to regulate calcium uptake by brain synaptosomes (Fraser *et al.*, 1988; Fraser and Sarnacki, 1988). The possibility that PTH has physiological roles within the CNS is also indicated by the finding that PTH IR is located in the central nervous system of fish, which lack peripheral parathyroid glands (Harvey *et al.*, 1987).

In summary, these results demonstrate that the PTH gene is transcribed in rat hypothalamic nuclei in regions that suggest roles for PTH in neurotransmission or neuroendocrine function.

Figure V-1. Plasmid constructs used in RNA probe synthesis. a) rPTHGEM4 containing exon III, intron B and part of exon II of the rPTH gene used for the synthesis of a 375 bp antisense RNA probe, after *Hind III* digestion, b) cPTHGEM2 containing the cPTH cDNA sequence used for the synthesis of a 430 bp sense RNA probe, after digestion with *Eco R1*.


Pst 1
intron B
rPTH gene
exon III
Bst N1
3'non-coding
Xba 1
T7 promoter
Hind III
rPTHGEM4
Eco R1
SP6 promoter


3'
Hind III
cPTH cDNA
T7 promoter
cPTHGEM2
Eco R1
SP6 promoter
5'
Bam H1

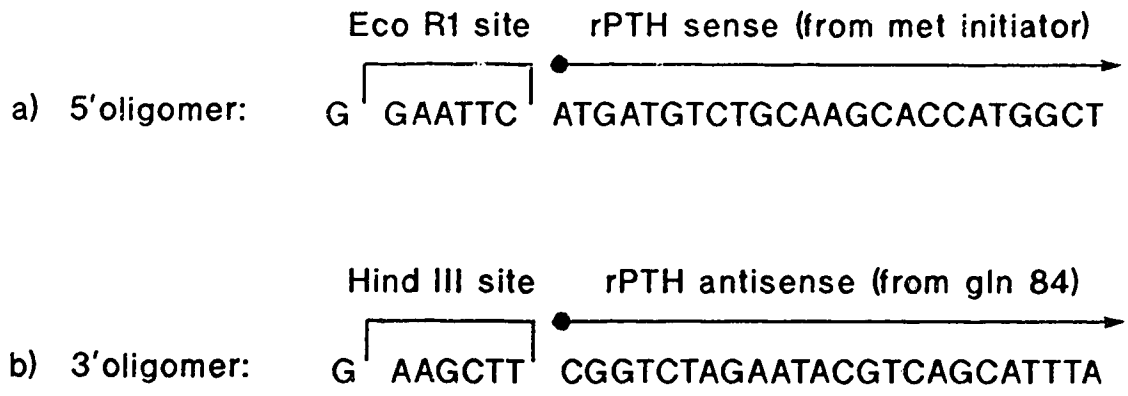


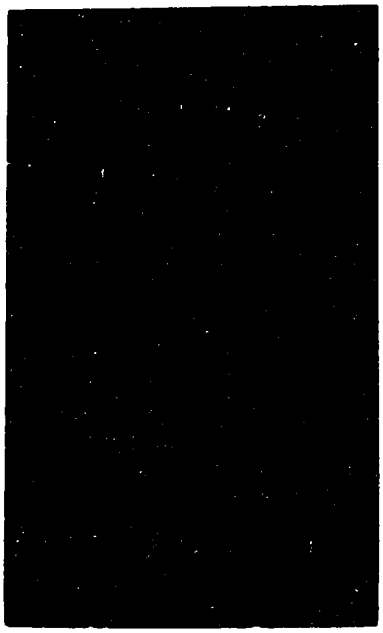
Figure V-2. Oligonucleotide primers used in the reverse transcription and PCR. The 5' oligomer primer a) is equivalent to the 5' end of the mRNA sequence, while the 3' oligomer primer b) is complementary to the 3' end of the mRNA sequence.

Figure V-3. Northern blot analysis of total RNA extracted from rat parathyroid gland (lane 1) and poly A^+ RNA from rat hypothalamus (lane 2), probed with antisense rPTH RNA probe. The migration of 18S RNA as viewed on ethidium bromide-stained gels, is indicated.

Figure V-4. Ethidium bromide stained gel of the *Hae* III digested pUC 18 low molecular weight markers (587, 456, 434, 298, 267, and 174, lane 1) and PCR amplified cDNA from parathyroidal (lane 2), liver (lane 3) and hypothalamic (lane 4) tissues.

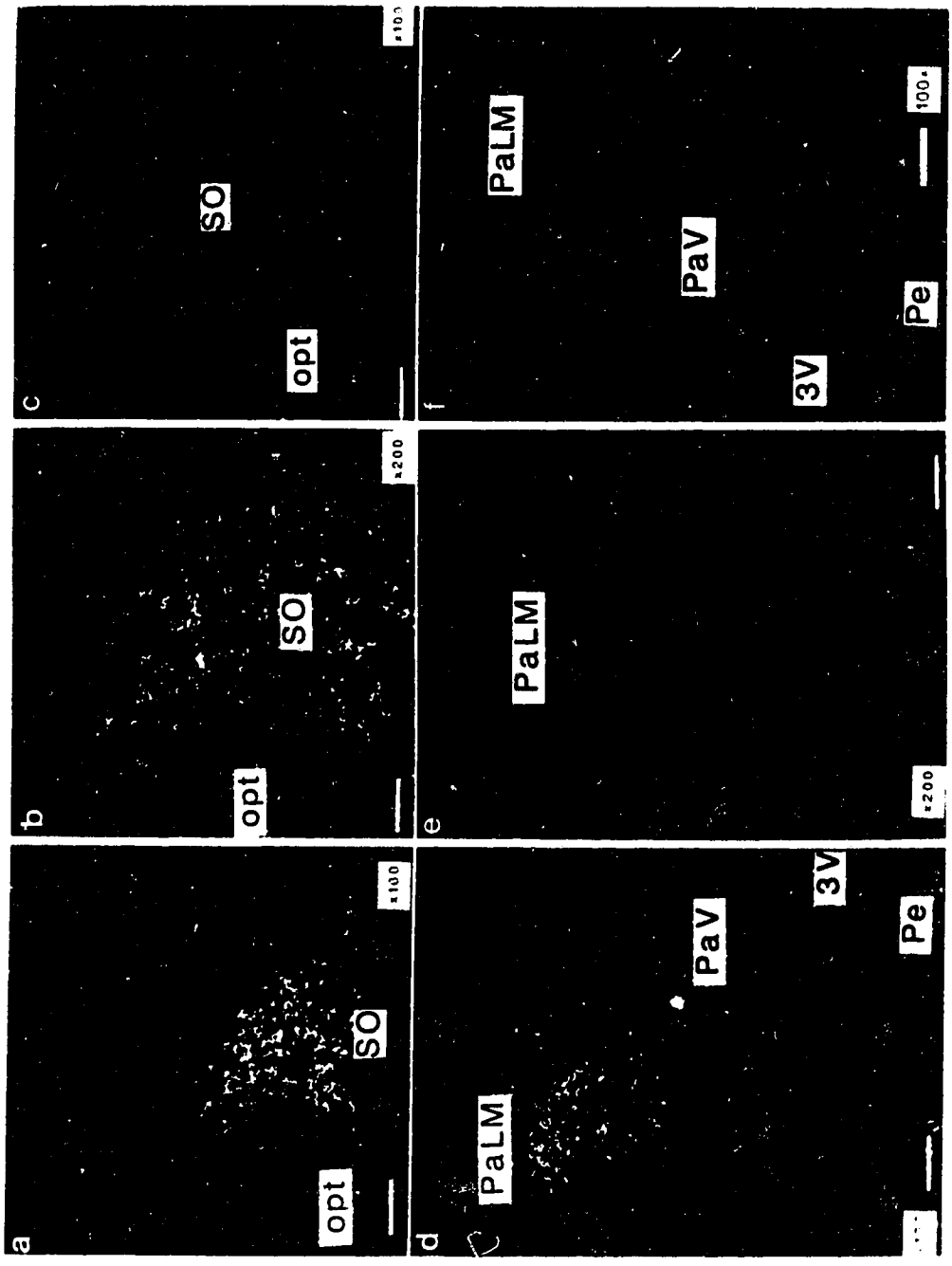
3

1 234



4

Figure V-5. Dark field photomicrographs of the *in situ* hybridization of rat brain cryostat sections with rPTH antisense (a, b, d and e) and cPTH sense (c and f) RNA probes. Accumulation of silver grains over the cells containing PTH mRNA appear as white clustering against the background. Paraventricular lateral magnocellular (PaLM), paraventricular (PaV) periventricular (Pe) and supraoptic nuclei (SO), as well as the third ventricle (3V) and optic tract (opt) are indicated in the micrograph. Bar = 50 μm and 25 μm for 100X and 200X magnification, respectively.


a
opt
SO
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b
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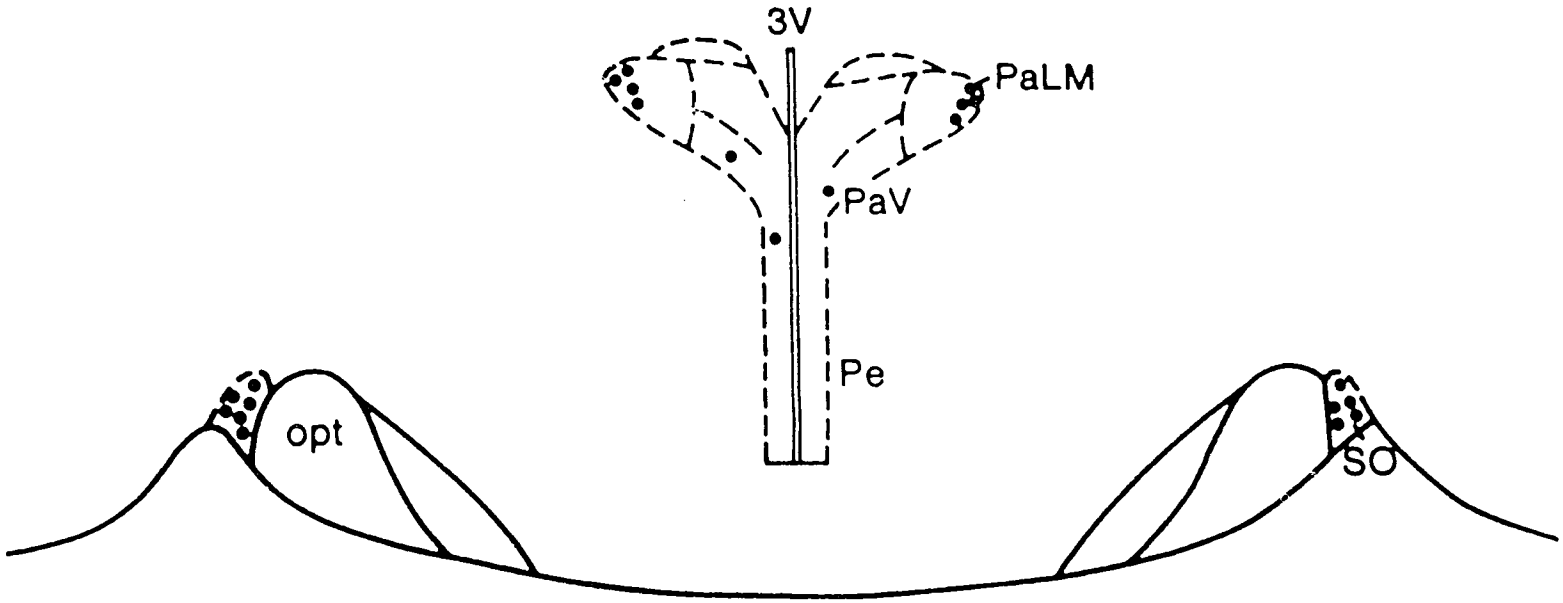

Figure V-6. Schematic drawing of a cross section through the rat's medial basal hypothalamus indicating the nuclei of highest rPTH mRNA content (●). Abbreviations as in Fig. V-5.

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CHAPTER VI

EXPRESSION OF PARATHYROID HORMONE-RELATED PEPTIDE GENE IN RAT HYPOTHALAMUS[1]

1. A version of this chapter has been submitted for publication. Fraser R, Zajac J, Kronenberg H, Harvey S, 1990 Expression of parathyroid hormone-related peptide gene in rat hypothalamus. Peptides.

INTRODUCTION

A novel parathyroid hormone-related peptide (PTHrp) has been purified and cloned from human malignant tumors associated with humoral hypercalcemia of malignancy (HHM) (Moseley *et al.*, 1987; Suva *et al.*, 1987). Eight of the first thirteen amino acids of PTHrp are identical to those of the parathyroid hormone (PTH) amino terminus, which is believed to give PTHrp its ability to bind to PTH receptors (Juppner *et al.*, 1988) and its potent activity in PTH bioassays (Goltzman *et al.*, 1989; Fraser, 1989; Orloff *et al.*, 1989). Similarities in tertiary structure (Barden and Kemp, 1989) may account for the ability of PTHrp to interact with PTH receptors and polyclonal antisera against PTH (Mundy and Martin, 1982).

The genes of both PTH and PTHrp are expressed in a variety of normal tissues (Ikeda *et al.*, 1988; Thiede and Rodan, 1988; Yasuda *et al.*, 1989; Weir *et al.*, 1990; Fraser *et al.*, 1990b). In particular, both peptides have been located in the rat central nervous system. However, while PTH mRNA has been localized in hypothalamic regions by *in situ* hybridization, Northern blot and polymerase chain reaction analysis (Fraser *et al.*, 1990b), PTHrp mRNA has been detected by *in situ* hybridization and ribonuclease (RNase) protection assay in extrahypothalamic regions (Weir *et al.*, 1990). The PTHrp gene may, nevertheless, be expressed in the hypothalamus, since close examination of the data presented by Weir *et al.* (1990) suggests the hybridization of

their PTHrp cDNA oligonucleotide probe with hypothalamic tissue sections is greater than the background signal. The possibility that PTHrp gene may be expressed in the rat hypothalamus has therefore been examined in the present study.

MATERIAL AND METHODS

Tissue Extraction

Hypothalami were rapidly dissected from decapitated 250 g male rats (Sprague-Dawley, Harlen Co., Indianapolis, IN) frozen on dry ice, and stored at -80^{o}C. The frozen tissue was then homogenized in 0.1 M HCL (10:1 vol:wt) centrifuged (1,000 x g, 4^{0}C), and the supernatant boiled and dialyzed as detailed previously (Harvey *et al.*, 1987). Lyophilized extracts were resuspended in RIA buffer [0.05 M PO_4, 0.16 M NaCl, 0.025 M disodium ethylene diamine tetraacetate (EDTA), 0.02% NaN_3 (wt:vol), 0.25% BSA (wt:vol) at pH 7.5] at a concentration of 10 mg/ml. For comparative purposes, rat pituitary glands and skeletal muscle and bovine parathyroid tissue from freshly killed animals were similarly processed.

Radioimmunoassay (RIA)

PTHrp immunoreactivity (IR) was determined in a double antibody RIA as previously described (Fraser *et al.*, 1990a); when used at a final dilution of 1:5000, the PTHrp-(1-34) antiserum (Suva *et al.*, 1987) bound 20% of the tracer with a sensitivity of <2.0 pg/ml PTHrp-(1-34). This assay is specific for PTHrp and has no cross-reactivity with PTH-(1-84) or PTH-(1-34) (Fraser *et al.*, 1990a).

RNA Preparation

Total RNA and polyadenylated RNA (mRNA) of rapidly dissected 250 g male Lewis rat hypothalami were prepared as outlined by Kingston *et al.* (1987). Briefly, the RNA pellets from isopicnic centrifugation through a 5.7 M $CsCl_2$ (containing 0.1 mM EDTA) bed were dissolved in 0.002 vol diethyl pyrocarbonate (DEPC)-treated water and phenol: chloroform (1:1 vol:vol) extracted twice, prior to application to a prepared oligodeoxythymidine-cellulose column. The eluted mRNA was ethanol (2.5 vol:vol), and 3 M sodium acetate (0.1 vol:vol) precipitated, then resuspended in DEPC-treated water. The mRNA was analysed by absorbance at 260 nm and ethidium bromide-stained 1% agarose gel. For comparative purposes, mRNA was prepared similarly from rat parathyroid cells (Sakaguchi *et al.*, 1987; Zajac *et al.*, 1989).

Northern Blot Analysis and Probe Synthesis

Polyadenylated RNA from rat hypothalami and parathyroid cells (5 μg) and total RNA from rat parathyroid cells (5 μg), used as a positive control (Ikeda *et al.*, 1988), were electrophoresed through a 1.4% agarose, 18% formaldehyde buffered (20 mM MOPS, pH 7.0, 1 mM EDTA, pH 8.0 and 5 mM sodium acetate, pH 5.2) gel (Maniatis *et al.*, 1982). The RNA was transferred to nitrocellulose by capillarity, and probed with random primed (Feinberg and Vogelstein, 1982) $^{32}P\alpha dATP$ (3,000 ci/mmol)-incorporated pBR50 (Suva *et al.*, 1987), containing the full length hPTHrp cDNA sequence, in 30% formamide, 5X SSPE (50 mM $NaH_2O_4H_2$), 750 mM NaCl, 0.5 mM EDTA,

pH 7.4), 5X Denhardt's (0.1% Ficoll, polyvinylpyrrolidine and BSA), 0.1% sodium dodecyl sulfate (SDS) and 100 μg/ml sheared salmon sperm DNA at 92°C for 12 h. The blot was then washed three times in 2X SSC (300 mM NaCl, 30 mM Na citrate, pH 7.0) containing 0.1% SDS at 42 °C for 30 min. Dried blots were exposed to X-OMAT AR film (Kodak, Rochester, NY) sandwiched between two intensifying screens for one day.

RESULTS

RIA

Serial dilutions of the rat hypothalamus and pituitary and the bovine parathyroid gland extracts over the range of 2.5, 1.25, 0.625, 0.312 and 0.15 mg/ml, displaced the binding of PTHrp-(1-34), ^{125}I-tyr^{34} to the PTHrp antisera in a manner parallel to PTHrp-(1-34), tyr^{34} standard (Fig. VI-1). The PTHrp IR in the extracts (determined at 50% displacement of binding) was 5 ng, 5.2 ng and 0.13 ng, respectively. Extracts of rat skeletal muscle had no PTHrp IR.

Northern Blot

As expected, hybridization of the PTHrp cDNA probe occurred with the rat parathyroidal cells. The hybridizing signal was intensified in the lane containing mRNA (Lanes 2 and 3, Fig. 2). The only other band detected was one of 1.8 kb that co-migrated with the parathyroidal cell RNA in the lane containing rat hypothalamic mRNA (Lane 1, Fig. VI-2).

DISCUSSION

PTHrp, originally purified and cloned from malignant tumors associated with HHM (Suva *et al.*, 1987; Mosely *et al.*, 1987), exists in normal tissues (Ikeda *et al.*, 1988; Yasuda *et al.*, 1989; Rodda *et al.*, 1988; Thiede and Rodan, 1988).

Recently, RNase-protection assay demonstrated PTHrp gene expression in the cerebellum, cerebrum, telencephalon, diencephalon extracts and brain stem, while *in situ* hybridization localized high concentrations of PTHrp mRNA in the hippocampal formation, cerebral cortex and cerebellum (Weir *et al.*, 1990).

The results presented in this study clearly indicate the presence of PTHrp and PTHrp mRNA in the rat hypothalamus. Although, it has been reported that cultured neuroendocrine cells synthesize and secrete PTHrp (Deftos *et al.*, 1989), this is the first report to conclusively show the presence of PTHrp. Although, the data presented by Weir *et al.* (1990) indicate the presence of PTHrp mRNA in hypothalamic tissues, this finding was not discussed, presumably because the low abundance of the message in comparison with other brain regions.

The presence of PTHrp IR in the hypothalamus suggests translation of the PTHrp message in hypophysiotropic regions in which PTH mRNA and PTH IR occur (Fraser *et al.*, 1990b; Pang *et al.*, 1988a; 1988b). PTHrp may therefore have a role in the regulation of pituitary function or neurocrine roles within the brain. The finding of PTHrp IR in the pituitary gland also resembles IR PTH distribution (Pang *et al.*, 1988a; 1988b), which is thought to indicate a

pituitary site of action or the neurohemal localization of the peptide prior to secretion into systemic circulation. The roles and regulation of PTH and PTHrp in the hypothalamopituitary axis have, however, yet to be determined.

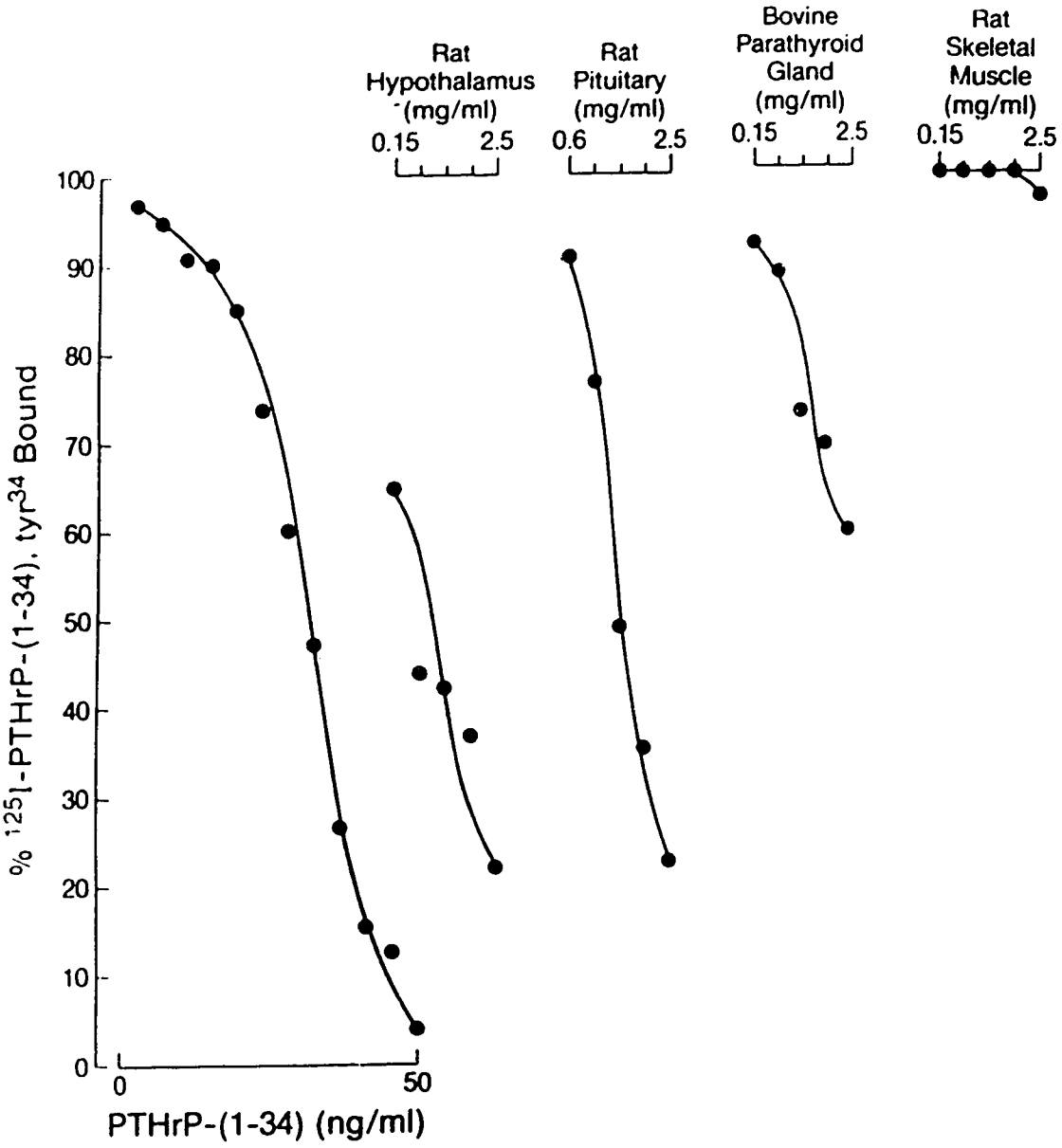
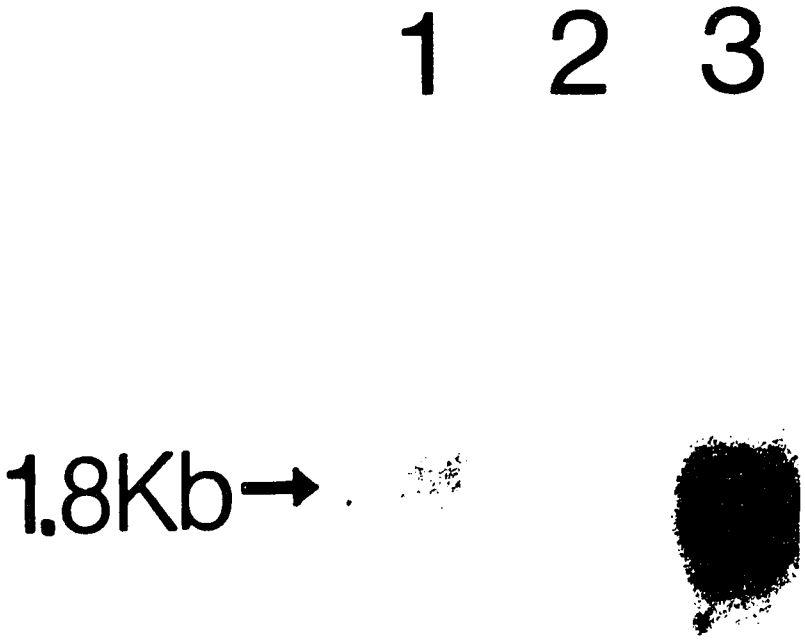


Figure VI-1. Cross-reaction of boiled dialyzed extracts of rat hypothalami and pituitaries and bovine parathyroid gland in PTHrp-(1-34) radioimmunoassay.

Figure VI-2. Northern blot analysis of polyadenylated RNA prepared from rat hypothalamus (lane 1) and parathyroid cells (lane 3) and total RNA from rat parathyroid cells (lane 2).


1 2 3
1.8Kb

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CHAPTER VII

GENERAL DISCUSSION

This series of immunoreactive (IR), chromatographic and molecular biological studies indicates the presence of parathyroid hormone (PTH), stanniocalcin and PTH related peptide (PTHrp) in the central nervous system (CNS) of vertebrate species. The localization of these peptide hormones in specific regions of the brain suggests they have central and/or peripheral roles.

CENTRAL PTH: ENDOCRINE ROLES?

Although encapsulated parathyroid glands first appear in vertebrate evolution in amphibian species (Rosenblatt *et al.*, 1989), immunoreactive (IR) PTH-(1-34), -(48-53) or -(53-64) has been measured in the serum of species lacking parathyroid glands, such as teleosts and mudpuppies (Table VII-1). Furthermore, injections of PTH systemically into fish induce hypocalcemic (Pang *et al.*, 1982; Lafeber *et al.*, 1986) and hypotensive actions (Pang *et al.*, 1980) suggesting the presence of PTH-like receptors in peripheral tissues. An endogenous endocrine with PTH-like activity would therefore appear to be present in lower vertebrates lacking parathyroid glands.

Almost every tissue and organ has been proposed as a putative parathyroid gland in fish (Table VII-2); the results of this thesis suggest the brain and/or the pituitary gland may be a source of PTH-like peptides in the peripheral plasma of fish and (perhaps) higher vertebrates. The detection of IR PTH-(1-34) and -(48-53) in pituitary extracts in protein fractions that co-elute with authentic hPTH (1-34) (Chapters III; IV) supports this view and is consistent

with the finding of IR PTH-(1-34) in nerve terminals in the adenohypophysis (Kaneko and Pang, 1987; Kaneko *et al.*, 1989). Since the pituitary is a neurohemal organ, PTH-like peptides synthesized in hypothalamic perikerya could therefore be released from the pituitary gland into systemic circulation (Fig. VII-I). Although IR PTH could not be demonstrated immunohistochemically in tetrapod pituitary glands (Pang *et al.*, 1988; Balabanova *et al.*, 1985), IR PTH in these tissues could easily be detected by radioimmunoassay and by HPLC purification of pituitary extracts (Chapters III; IV). The pituitary may therefore also secrete PTH-like peptides into peripheral circulation in higher vertebrates. The localization of PTH IR and mRNA in the paraventricular and supraoptic nuclei of vertebrate brains (Chapter IV; V; Pang *et al.*, 1988; Balabanova *et al.*, 1985) supports this conclusion, since these nuclei have axons projecting into the neurohypophysis from which oxytocin and vasopressin are released into the bloodstream (Scharrer and Scharrer, 1954). The pulsatile release of PTH-like peptides from the neurohypophysis into systemic circulation is, moreover, suggested by the demonstration that the concentrations of IR PTH in the jugular vein exceed those in hypophysial portal plasma, which is derived from blood supplying the brain (Appendix I). The possible contributions of IR PTH in peripheral plasma is also suggested by the measurement of amino-terminal and mid-region IR PTH in the plasma of parathyroidectomized patients (Goltzman *et al.*, 1984).

In addition to the pituitary gland, PTH-like peptides synthesized in the brain may enter peripheral plasma via the superior saggital sinus and arachnoid villus, which provide an interface between neural extracellular fluids and the cardiovascular system (Bradbury, 1979). This possibility is supported by the presence of IR PTH in human cerebrospinal fluid at concentrations similar to those in peripheral plasma (Balabanova *et al.*, 1984; Saggese *et al.*, 1986).

If PTH and/or PTH-like peptides of neural origin do contribute to circulating PTH concentrations, these peptides may have "classical" endocrine roles at distant target sites. Since the release (but not content, Chapter IV) of IR PTH from the brain is stimulated *in vitro* by lowered calcium levels and suppressed by $1,25(OH)_2$Vitamin D_3 (Balabanova *et al.*, 1986), similar to the release of PTH from the parathyroid glands (Rosenblatt *et al.*, 1989), neural PTH or PTH-like peptides may play an endocrine role in calcium homeostasis.

CENTRAL PTH: NEUROENDOCRINE ROLES?

Parathyroid hormone IR and mRNA are in brain nuclei which, in turn, are involved in hypophysiotropic regulation (Chapter II; IV; V; Harvey *et al.*, 1987; Kaneko and Pang, 1987; Pang *et al.*, 1988). Moreover, IR PTH fibres from the paraventricular and supraoptic nuclei in mammals and preoptic nuclei in other terrestrial vertebrates, terminate around hypophysial portal blood vessels, in the external zone of the median eminence (Pang *et al.*, 1988; Kaneko *et al.*, 1989). A hypophysiotropic role for PTH or

PTH-like peptides of central origin is therefore suggested. This possibility is further supported by the finding of PTH IR nerve terminals in the pituitary gland of teleosts, in which hypophysial portal vessels are lacking and adenohypophysial function is directly regulated by the neural innervation of the pituitary gland (Peter, 1986; Schreibman, 1986). The positive correlation between circulating PTH and prolactin levels in hyperparathyroidism (Issac *et al.*, 1978; Castro *et al.*, 1980; Brickman *et al.*, 1981; Kruse *et al.*, 1981; Raymond *et al.*, 1982; Raymond *et al.*, 1982) provides some evidence for a putative hypophysiotropic role for neural PTH, especially as circulating prolactin levels decline following parathyroidectomy (Raymond *et al.*, 1982) and exogenous PTH and parathyroid extracts increase plasma prolactin concentrations in man (Issac *et al.*, 1978; Castro *et al.*, 1980; Kruse *et al.*, 1987; Raymond *et al.*, 1981; Brickman *et al.*, 1981). PTH does not, however, appear to have any direct effects on the basal or stimulated release of prolactin or other adenohypophyseal hormones from incubated rat pituitary glands or tumorous rat pituitary cell lines (GH_3 cells) (S. Harvey, unpublished observations).

If PTH is acting as a neuroendocrine hormone, this would be in accordance with other calcium-regulating hormones, such as calcitonin (CT) and Vitamin D_3 (Vit D). Receptors for CT and Vit D occur in the pituitary and hypothalamic tissue (Haussler *et al.*, 1982; Shah *et al.*, 1990). Furthermore, central CT has been shown to decrease basal and stimulated growth hormone release *in vivo* and

basal and stimulated prolactin release *in vitro* (Shah *et al.*, 1990) and Vit D has been shown to increase basal and stimulated TSH and prolactin release *in vivo* and *in vitro* and to inhibit GH release *in vitro* (Torquist and Tashijian, 1989; d'Emden and Wark, 1988).

CENTRAL PTH: NEUROCRINE ROLES?

The presence of PTH in the brain suggests it may be involved in central neurotransmissions. This is supported by the β-adrenergic-like activities of PTH in cardiac and smooth muscle tissue (Pang *et al.*, 1986). Furthermore, PTH actions on neural tissues have also been demonstrated including inhibition of L-type calcium conductance in neuroblastoma cells (Pang *et al.*, 1990), regulation of the Na^{+}/Ca^{++} exchanger in brain synaptosomes (Fraser *et al.*, 1988; Fraser and Sarnacki, 1988) and inhibition of one or more CNS areas responsible for gastric activity (Clementi *et al.*, 1989). PTH has also been shown to specifically increase dopamine metabolism in the rat hypothalamus (S. Harvey, personal communication), supporting a neurocrine role for this peptide (Fig. VII-1). An increase in the metabolism of dopamine may therefore account for the stimulatory effect of exogenous PTH on prolactin secretion (Blum *et al.*, 1980), since dopamine exerts inhibitory control over prolactin release (Moore, 1987).

Central dysfunctions in neurotransmission are also well-established symptoms of primary hyperparathyroidism and of uremia in which PTH secretion is secondarily elevated (Fraser *et*

al., 1988; Fraser and Sarnacki, 1988). Although these lesions were once thought to reflect disturbances in calcium homeostasis, Fraser and his colleagues (1988) have now shown that PTH has direct effects on neural physiology and is likely to be responsible for the central dysfunctions in these pathophysiological states. This possibility is also supported by the presence of PTH-binding sites on the plasma membranes of the rat brain and the effects of PTH on catecholamine metabolism (S. Harvey, unpublished observations).

The binding sites for PTH in the brain are, however, unlikely to be occupied by PTH of peripheral origin under normal conditions. Although PTH can cross the blood-brain-barrier (Care, 1987), entry of PTH into the brain from systemic circulation is likely to be minimal (Partridge, 1987), except in hyperparathyroid syndromes. PTH of central origin is therefore normally likely to act at these binding sites in a neurocrine manner.

CENTRAL PTH: AN ANCESTRAL NEUROPEPTIDE?

It is now established that PTH IR and mRNA are present in the CNS of vertebrates and at least one invertebrate (Wendelaar Bonga *et al.*, 1989). The presence of central PTH in species with and without encapsulated parathyroid glands suggests an ancestral neuron may have been the origin of this peptide. The immunocytochemical staining of amino-terminal hPTH in gastropod sensory ganglia suggests a rimary role for PTH in neurotransmission (Wendelaar Bonga *et al.*, 1989). The participation of PTH in calcium homeostasis may have evolved later in the vertebrates as an evolution from local

neurotransmitter control of ligand-gated calcium channels in invertebrates to whole body calcium homeostasis in vertebrates.

For PTH to induce bioactivity, a receptor-mediated mechanism is a prerequisite (Roth *et al.*, 1982). It is therefore axiomatic that the evolution of a peptide must occur contemporaneously with or after the evolution of its receptor (Joose, 1987). The proposed occurrence of PTH receptor isoforms (Rosenblatt *et al.*, 1989) suggests the continuation of this evolutionary process and may account for the various activities induced by PTH throughout phylogenetic development. Moreover, the primary, secondary and tertiary structure of PTH has been relatively well conserved, as evidenced by the homology of the amino acid sequences (Fig I-6) and the ability of mammalian PTH antibodies to cross-react with putative PTH-peptides in lower vertebrates (Pang *et al.*, 1988; Kaneko and Pang, 1987; Harvey *et al.*, 1987; see Table VII-2) and invertebrates (Wendelaar Bonga *et al.*, 1989). Furthermore, although the sequence is unknown, homology between amphibian and mammalian PTH sequence is suggested by the presence of an mRNA in bullfrog parathyroid glands that cross hybridizes with chicken and human cDNA sequences and is similar in size to mammalian PTH mRNA (Appendix II). However, it has not been determined whether sequence differences between neuronal and parathyroid glandular PTH exist, possibly as a result of a divergent evolutionary pathway. This is unlikely, however, given the size similarity of neuronal and parathyroidal PTH in Northern blot and PCR analysis (Chapter IV) and

the fact that only one rat PTH gene has been detected (Heinrich *et al.*, 1984).

STANNIOCALCIN: A PTH-LIKE PEPTIDE?

A major component of the corpuscles of Stannius (CS), stanniocalcin, has been purified (Wagner *et al.*, 1986; 1988; Lafeber *et al.*, 1988) and cloned (Butkus *et al.*, 1987) from teleostean species and is believed to have hypocalcemic or antihypercalcemic activities (Wagner *et al.*, 1986; 1988; Lafeber *et al.*, 1988; Wendelaar Bonga *et al.*, 1990). The purification of stanniocalcin was based on the PTH-like hypercalcemic activity in mammalian (Lafeber *et al.*, 1986) and hypocalcemic activity in teleostean bioassays (Wagner *et al.*, 1986; 1988; Lafeber *et al.*, 1988). Although controversial, immunocytochemical staining (Milet *et al.*, 1982; MacIntyre *et al.*, 1981; Lopez *et al.*, 1982a; 1982b; 1984a; 1984b; Orimo *et al.*, 1982) and radioimmunoassay of CS and CS extracts (Harvey *et al.*, 1987) demonstrated the presence of IR amino- and/or carboxyl-terminal PTH. However, no such cross-reactivity of antibodies raised against PTH occurred with purified stanniocalcin (Chapter III; Wagner *et al.*, 1986; 1988 Butkus *et al.*, 1987; Lafeber *et al.*, 1988) and no cross-reactivity of antibodies raised against stanniocalcin occurred with PTH (Chapter II; III). Furthermore, despite similar bioactivities, no sequence homology exists between the species of stanniocalcin purified and known PTH sequences (Wagner *et al.*, 1986; 1988; Butkus *et al.*, 1987;

Lafeber *et al.*, 1988). IR PTH measured by others in CS extracts and tissues is therefore likely to be distinct from stanniocalcin, unless different tissue fixation or extraction procedures of CS alter stanniocalcin tertiary structure, such that PTH-raised antibodies may then cross-react (Krieger *et al.*, 1985).

Although stanniocalcin and CS extracts have been demonstrated to be potent antihypercalcemic factors (Table VII-2; Wagner *et al.*, 1988; Wendelaar Bonga *et al.*, 1990) and, therefore, antagonistic to the hypercalcemic effects of prolactin, other factors, including PTH, PTHrp and "parathyrin of the CS" (Milet *et al.*, 1990) may also be involved in lowering fish serum calcium levels. Since stanniocalcin release from the CS *in vitro* requires extremely high calcium levels (2.5-3.5 mM) (Hanssen *et al.*, 1990), other factors are likely to respond to smaller increases in the circulating calcium concentration.

STANNIOCALCIN: A NEUROPEPTIDE?

The distribution of IR stanniocalcin in brain regions in which IR PTH has been located suggests novel endocrine, neuroendocrine or neurocrine roles for IR stanniocalcin (Fig. VII-1; Chapter III), as for PTH.

Stanniocalcin of peripheral origin has established endocrine roles in peripheral target tissues, particularly the inhibition of calcium fluxes across the branchial epithelium (Verbost *et al.*, 1990; Lafeber *et al.*, 1988; 1989; Wagner *et al.*, 1986; 1988; So and Fenwick, 1979; Milet *et al.*, 1979). Endocrine roles for

central stanniocalcin, if it enters peripheral circulation, have yet to be described.

A neuroendocrine role for central stanniocalcin is, however, suggested by the stimulation of prolactin release and synthesis by intraperitoneally injected CS extract (Srivastav *et al.*, 1987). Although prolactin is hypercalcemic in fish (Parsons *et al.*, 1978; Pang *et al.*, 1978), CS-induced prolactin release may reflect a fine-tuned mechanism to prevent excessive hypocalcemia.

The stimulation of stanniocalcin release by acetylcholine agonists (Hanssen *et al.*, 1990), suggests that neural control over CS exists. Centrally located stanniocalcin may play a role in CS activity via peripheral afferent innervating axons, since the CS is a highly innervated tissue (Wendelaar Bonga and Pang, 1986).

The striking similarities in IR PTH and stanniocalcin distribution in the CNS, suggests that the two systems may be related. Perhaps IR stanniocalcin and PTH are released and act together on the same or different target tissues in regulating calcium. It is also possible that peptide interaction between the two exists and contributes to calcium homeostasis. The release of stanniocalcin from the CS is induced by nerve-like stimulation (Hanssen *et al.*, 1990), which may reflect a role for central IR PTH on CS regulation, since PTH has β-adrenergic like effects in mammals (Pang *et al.*, 1986).

STANNIOCALCIN: AN ANCESTRAL NEUROPEPTIDE?

Stanniocalcin IR has also been detected in the sensory ganglia of the invertebrate CNS, together with IR PTH (Wendelaar Bonga *et al.*, 1989). The lack of CS in the invertebrate species (Copp and Ma, 1980), indicates that this peptide may have a neuronal origin.

Although stanniocalcin has demonstrated PTH-like activity in mammalian bone calveria (Lafeber *et al.*, 1986), no IR stanniocalcin has been determined in mammalian plasma or in a variety of tested tissues (unpublished observation). Therefore, IR stanniocalcin may not have been evolutionarily conserved in terrestrial vertebrates despite the possible presence of conserved stanniocalcin receptors (Lafeber *et al.*, 1986). Alternatively, extracted teleostean stanniocalcin may have enough tertiary structural homology to interact with PTH receptors to perform in a PTH-like manner.

PTH-RELATED PEPTIDE

The original isolation and cloning of PTHrp was from human tumor cells associated with humoral hypercalcemia of malignancy (Moseley *et al.*, 1987; Suva *et al.*, 1987). This peptide is, however, also found in a variety of normal tissues (Ikeda *et al.*, 1988; Yasuda *et al.*, 1989. Rodda *et al.*, 1988; Thiede *et al.*, 1988; Thiede and Rodan, 1988) and in particular the rat brain (Weir *et al.*, 1990; Chapter VI). The distribution of PTHrp in the rat brain is most concentrated in the hippocampal formation and cerebral

cortex (Weir *et al.*, 1990), although it is also present in other regions including hypothalamic areas (Chapter VI).

The functional role of PTHrp in the hippocampal regions is believed to be one of neurotransmission and local calcium regulation (Weir *et al.*, 1990). This possibility is supported by the high level of neural activity and of calcium antagonist binding proteins, (believed to be L-type channels) found in these regions (Weir *et al.*, 1990).

The functional role of PTHrp in the hypothalamus is thought to be similar to those proposed for PTH, since PTHrp can perform well in PTH bioassays (Goltzman *et al.*, 1989; Fraser 1989; Orloff *et al.*, 1989) and binds with PTH receptors (Juppner *et al.*, 1988). Therefore, PTHrp may be a neurocrine, neuroendocrine or endocrine peptide (Fig. VII-1), the latter being a possible source for PTHrp in blood serum (Gaich and Burtis, 1990). However, most evidence of PTHrp expression suggests that PTHrp exerts a paracrine or autocrine action, and levels of circulating PTHrp are due to pathologies such as malignant tumors (Weir *et al.*, 1990).

EVOLUTION OF PTHRP

Unlike stanniocalcin, PTHrp has some sequence homology with PTH, especially in the first 13 amino-acids of the amino-terminus (~60%). Furthermore, the prohormone cleavage site is retained in all known species of PTHrp and PTH (Fig. VII-2).

The human PTHrp gene and PTH gene have been localized on chromosome 12 (Mangin *et al.*, 1989) and chromosome 11 (Naylor

et al., 1983), respectively; these chromosomes bear other related genes and may have arisen from a single ancestral chromosome. Both genes do, however, share some common organizational features. The 5′ untranslated region of both genes is encoded by a single exon that is joined to a second exon coding for the prepro-region of the precursor peptide. The lys-arg prohormone cleavage site is then spliced to the prepro-containing exons which also contain the 3′ untranslated region (Fig. VII-2). Unlike the PTH gene, PTHrp undergoes alternative splicing (Mangin *et al.*, 1989; Yasuda *et al.*, 1989a) which produces multiple mRNA species in a variety of different tissues (Ikeda *et al.*, 1989). Although differences in sequence and the number of exons occur between PTH and PTHrp, chromosomal localization, similarities in structural organization and a block of sequence homology (amino acids 1-13) suggest that PTHrp and PTH may have been derived from an ancestral gene. Since distinct PTH and PTHrp IR is present in fish species, it is likely that a mutation of the ancestral gene occurred very early in time.

A NOVEL "FAMILY" OF PTH-LIKE NEUROPEPTIDES?

Many brain-gut peptides, it is now known, belong to super families, such as the members of the glucagon-secretin family (Said, 1984). The result of this present thesis suggest that a novel PTH-like neuropeptide family, to which PTH, PTHrp and stanniocalcin are members, also exists. These PTH-like peptides appear to have similar or complementary actions in regulating calcium metabolism throughout the vertebrates (Rosenblatt *et al.*, 1989; Goltzman

et al., 1989; Lafeber *et al.*, 1986; Pang *et al.*, 1980) in mammalian and teleostean bioassays. Although PTH has no apparent sequence homology with stanniocalcin (Wagner *et al.*, 1986; 1988; Butkus *et al.*, 1987; Lafeber *et al.*, 1989), certain moeities are believed to have resemblance in their tertiary structure (Pang *et al.*, 1980; Lafeber *et al.*, 1986; Wendelaar Bonga and Pang 1984; Ma and Copp, 1989; Milet *et al.*, 1981; Lopez *et al.*, 1982a; 1982b; 1984; Orimo *et al.*, 1982; Harvey *et al.*, 1987). Similarly, PTH and PTHrp have limited amino acid sequence homology in the first 13 amino-terminal residues (Suva *et al.*, 1987), but the amino-terminal portions of these peptides share tertiary structures as determined by nuclear magnetic resonance (Barden and Kemp, 1989). PTH and PTHrp gene structure is, furthermore, also similar (Mangin *et al.*, 1988; Yasuda *et al.*, 1988), as is the distribution of PTH, PTHrp and stanniocalcin in the CNS, particularly in hypophysiotropic regions of the hypothalamus (Chapter II; IV; V; IV). Therefore, based on the functional, structural and distributional similarities of its members, a PTH-like family of neuropeptides may exist.

SUMMARY

The presence of IR PTH, stanniocalcin and PTHrp as well as the mRNA of PTH and PTHrp in the CNS of vertebrate species, introduced three novel neural peptidergic systems. Although, the exact roles of these neuropeptides remains to be determined, their localization within certain regions of the CNS suggests certain endocrine,

neuroendocrine and neurocrine roles. The presence of these related peptides in nerve tissue suggests that PTH, PTHrp and stanniocalcin may have evolved from ancestral neurons and that these peptides collectively constitute a family of neuropeptides.

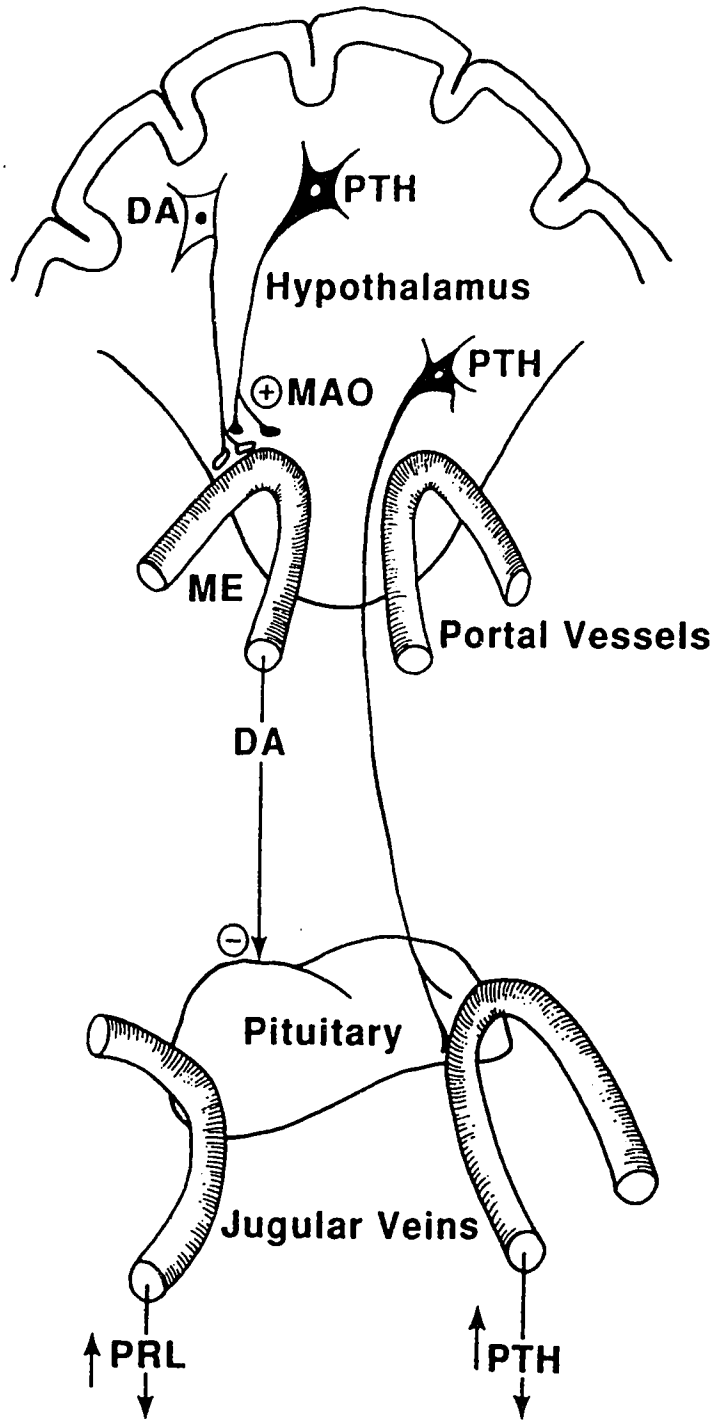
Table VII-1: Immunoreactive parathyroid hormone in the plasma of species lacking parathyroid glands.

Specificity of PTH Antisera	Species	Reference
N-terminal	European eel	Milet *et al.*, 1980; 1982; 1985
N-terminal	European eel	MacIntyre *et al.*, 1981
N-terminal	European eel	Lopez *et al.*, 1982a; 1982b; 1984a; 1984b
COOH-terminal	Japanese eel	Orimo *et al.*, 1982
COOH-terminal	Chum salmon	Orimo *et al.*, 1982
COOH-terminal	Trout	Harvey *et al.*, 1987
COOH-terminal	Goldfish	Harvey *et al.*, 1987
COOH-terminal	Mudpuppies	Pang *et al.*, 1988

Table VII-2. Putative "parathyroid gland" in fish

Tissue	Reference
Ultimobranchial Body	Copp and Ma, 1980 Rasquin and Rosenbloom, 1954
Pineal Gland	Pang, 1971; Rasquin and Rosenbloom, 1954
Thyroid Gland	Pang and Pickford, 1967
Ovaries	Balbontin *et al.*, 1978; Watts *et al.*, 1975
Interrenal Gland	Vargus and Concha, 1957
Head Kidney	Kenny *et al.*, 1977; Orimo *et al.*, 1982
Corpuscles of Stannius	Fontaine 1964; Chan *et al.*, 1969; Milet *et al.*, 1980; 1982
Pituitary Gland	Fontaine 1956; Parsons *et al.*, 1978; Pang *et al.*, 1982; Harvey *et al.*, 1987; Lopez *et al.*, 1984b
Brain	Harvey *et al.*, 1987; Pang *et al.*, 1988a; 1988b

Figure VII-1. Schematic diagram depicting potential effects of parathyroid hormone (PTH) within the hypothalamo-hypophysial axis. Neurons in the paraventricular or supraoptic nuclei synthesizing PTH may release PTH directly into portal vessels from axons terminating in the median eminence (ME) or jugular vessels from axons terminating in the neurohypophysis, for neuroendocrine and endocrine functions, respectively. A possible neurocrine mechanism based on recent findings (S. Harvey, personal communication), whereby PTH may regulate dopamine (DA) metabolism through the activation of monamine oxidase (MAO), is presented. It is proposed that PTH may indirectly stimulate prolactin (PRL) secretion from the adenohypophysis by removing DA inhibition or by direct PTH stimulation of PRL secretion, arriving through the portal system. In the absence of portal vasculature, a similar system whereby PTH arrives in the neurohypophysis in fish to act on the adenohypophysis may be operating (see text). By similar mechanisms, it is suggested that stanniocalcin and PTH-related peptide may also operate in this system (see text for details).


DA
PTH
Hypothalamus
⊕MAO
PTH
ME
Portal Vessels
DA
⊖
Pituitary
Jugular Veins
PRL
PTH

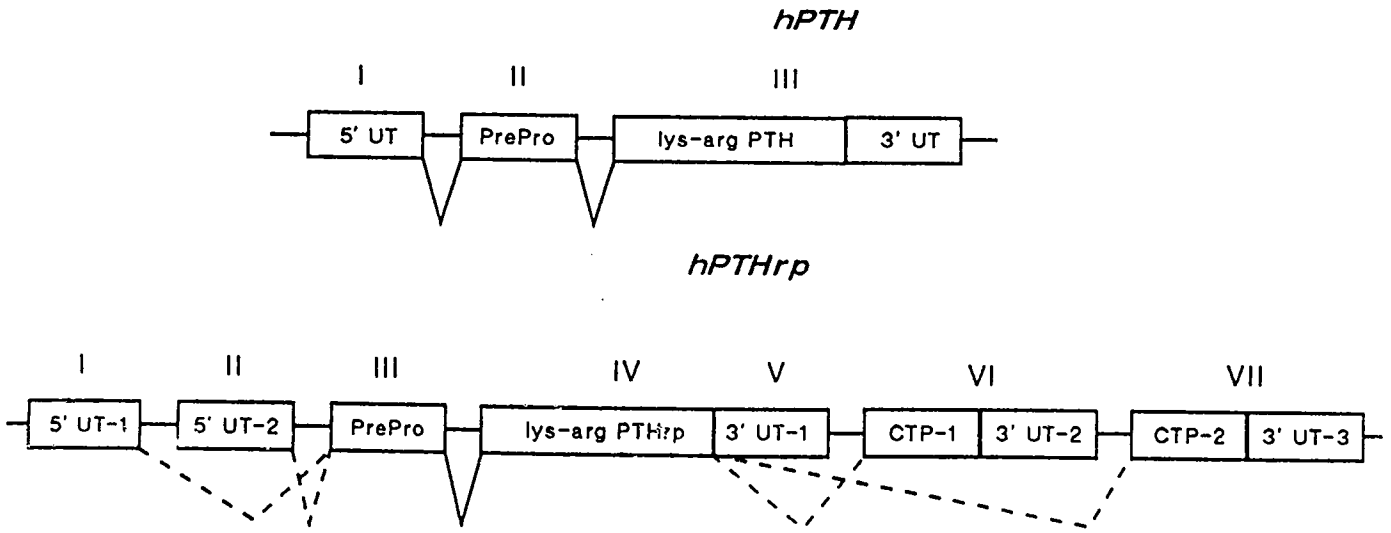


Figure VII-2. Comparative organization of human parathyroid hormone (hPTH) and hPTH-related peptide (hPTHrp) genes. Numbered boxes represent exons. Exons in the hPTHrp gene, which are joined by solid lines, are used constitutively whereas exons joined by dashed lines are combinatorial. In both hPTH and hPTHrp genes, the first exon used contains the 5' untranslated (UT) regions, the second exon used contains most of the prepro coding sequence, and the third exon contains the lys-arg prohormone cleavage site and all (in the case of PTH) or most (in the case of PTHrp) of the region encoding the mature peptide. In the case of PTHrp, alternative carboxyl-terminal peptides (CTP-1 and CTP-2) and alternative 3' untranslated (UT) regions also exist (adapted from Goltzman et al., 1989).

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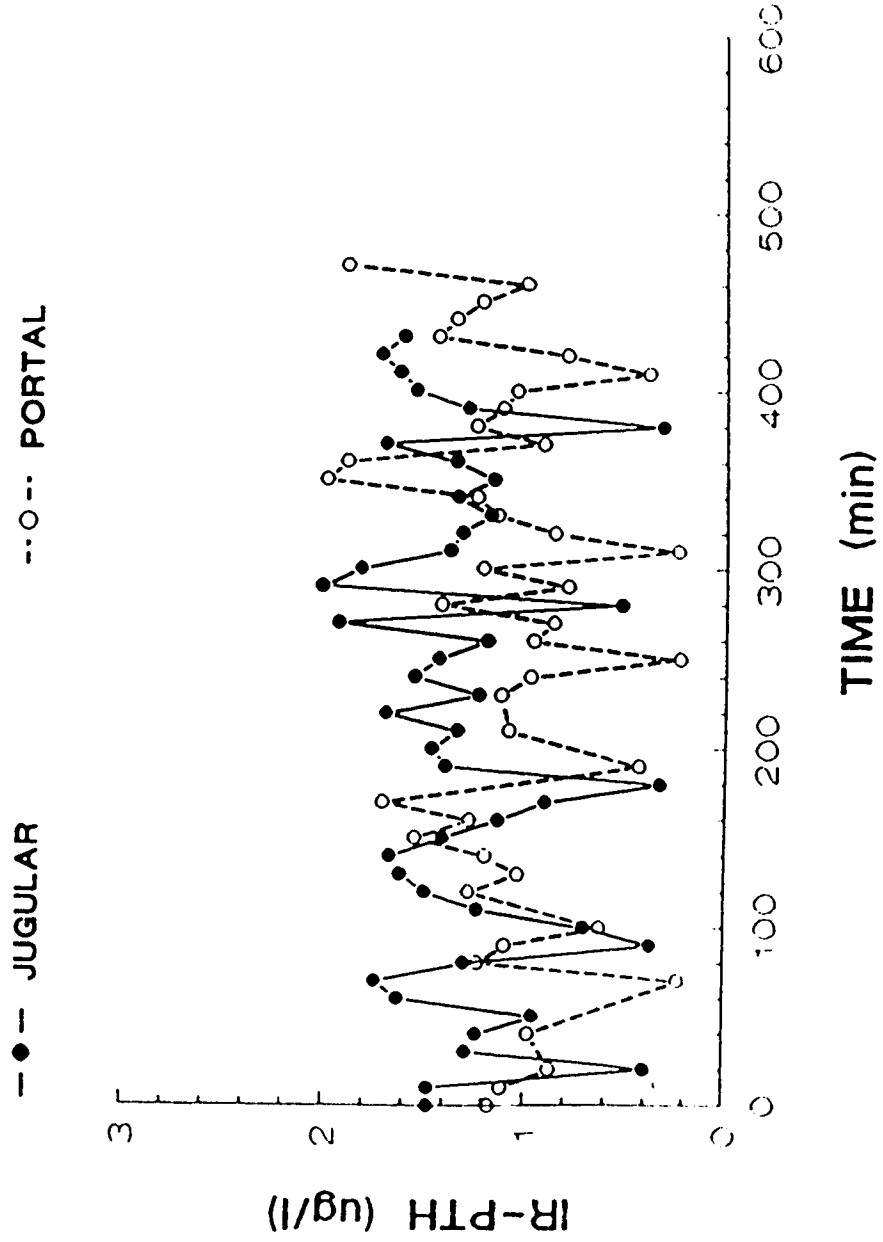
A P P E N D I X I

IMMUNOREACTIVE PARATHYROID HORMONE IN HYPOPHYSIAL PORTAL AND JUGULAR PLASMA.

Plasma immunoreactive PTH (53-64) in samples simultaneously taken from [illegible] physial portal and jugular vessels of sheep. Means (n=3).

[illegible] was provided by Dr. F.J. Karsh, Reproductive Sciences Program, The University of Michigan.

The data indicate a phasic release of immunoreactive PTH in both the portal and jugular vessels at levels higher than those in peripheral vessels.

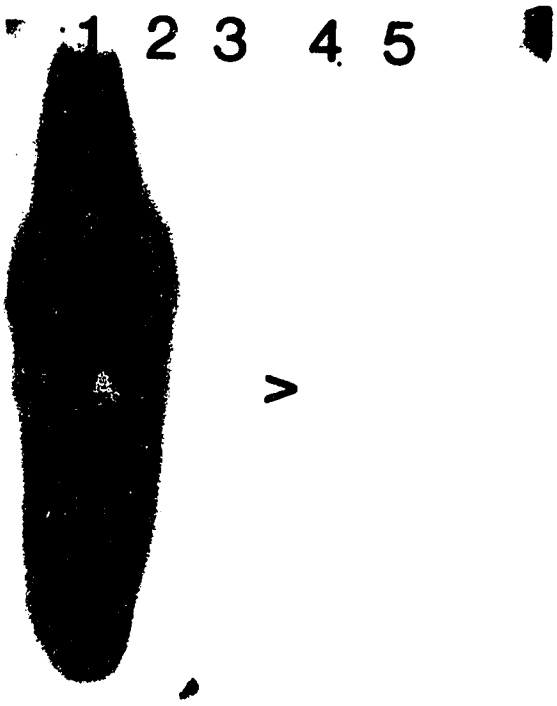

—●— JUGULAR
--○-- PORTAL
IR-PTH (ug/l)
0
1
2
3
0
100
200
300
400
500
600
TIME (min)

A P P E N D I X I I

DETECTION OF BULLFROG PTH MESSENGER RIBONUCLEIC ACID.

Total ribonucleic acid (RNA) extracted from chicken parathyroid gland (Lane 1), frog liver (Lane 2) and skin (Lane 3), rat parathyroid gland (Lane 5) and polyadenylated ribonucleic acid (mRNA) extracted from frog parathyroid glands (Lane 4), as previously described (Chapter V, VI), was analysed by Northern blot (Maniatis *et al.*, 1982) with random primed (Chapter VI) $^{32}P\alpha ATP$ (3000 Ci/mmol) chicken (Khosla *et al.*, 1988) cDNA probe. The blot was washed three times in 2X SSC and 0.1% SDS 42°C.

A band of similar size to rat PTH mRNA hybridized with the chicken cDNA probe in the lane 4 containing frog parathyroid gland mRNA.


1 2 3 4 5

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APPENDIX III

PUBLICATIONS

The figures and tables in this thesis have appeared previously in at least one of the following publications and/or abstracts:

Refereed Papers

1. Kaneko T, Fraser RA, Labedz T, Harvey S, Lafeber FPTG, Pang PKT, 1988 Characterization of antisera raised against hypocalcin (teleocalcin) purified from the corpuscles of Stannius of rainbow trout, *Salmon gairdneri*. Gen Comp Endo **69**: 238-245.
2. Fraser RA, Kaneko T, Pang PKT, Harvey S, 1990 Hypo-and hypercalcemic peptides in fish pituitary glands. Am J Physiol (in press).
3. Pang PKT, Harvey S, Fraser R, Kaneko T, 1988 Parathyroid hormone-like immunoreactivity in brains of tetrapod vertebrates. Am J Physiol 225: R635-R642.
4. Fraser RA, Kronenberg HM, Pang PKT, Harvey S, 1990 Parathyroid hormone mRNA in the rat hypothalamus. Endocrinology (in press).

Abstracts

1. Fraser R, Kaneko T, Harvey S, Pang PKT, 1987 Immunoreactive parathyroid hormone (IR-PTH) in brain and gut of vertebrate species: novel peptidergic systems? Symposium on Molecular Biology of Brain and Endocrine Peptidergic Systems (Gene Expression and Biomedical Applications), Montreal, Quebec, Oct 13-16.
2. Kaneko T, Harvey S, Fraser RA, Pang PKT, 1987 Studies on a PTH-like substance in the Stannius corpuscles of fish. First International Conference on New Actions of Parathyroid Hormone, Kobe, Japan Oct 27-31.

3. Fraser RA, Harvey S, Pang PKT, 1988 Characterization of parathyroid hormone-like immunoreactivity in neural and non neural tissues. The Endocrine Society 70th Annual Meeting, New Orleans, LA, June 8-11.

4. Fraser RA, Harvey S, Kaneko T, Pang PKT 1989 The detection of PTH-like peptide messenger RNA in rat hypothalamus by *in situ* hybridization. XIth International Symposium on Comparative Endocrinology, Malaga, Spain, May 15-21

Although my name does not appear as the senior author in papers 1 and 3, I have included the data from these papers in my thesis since I was responsible for producing most (if not all) of the figures in paper 1 (Chapter II) and figures 5, 7 and 8 in paper 3 (Chapter IV). The other figures in paper 3 (Chapter IV) are in my thesis as I have repeated the experiments and presented the identical findings in abstracts 1, 2 or 3.