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CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P IN THE BOVINE PARATHYROID GLAND: IMMUNOHISTOCHEMICAL LOCALIZATION AND EFFECT ON PARATHYROID HORMONE SECRETION

by

SHANE T. MORTIMER

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- General 0318
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American Studies 0323
Anthropology
- Archaeology 0324
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Business Administration
- General 0310
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Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology ... 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

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Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

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Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
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Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

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Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology .. 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

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- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics ... 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY

General 0621
Behavioral 0384
Clinical 0622
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Industrial 0624
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Physiological 0989
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Art 0273
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Enseignement religieux 0527
Enseignement secondaire 0533
Enseignement spécial 0529
Enseignement supérieur 0745
Évaluation 0288
Finances 0277
Formation des enseignants 0530
Histoire de l'éducation 0520
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- Moderne 0298
- Africaine 0316
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- Anglaise 0593
- Asiatique 0305
- Canadienne (Anglaise) 0352
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- Germanique 0311
- Latino-américaine 0312
- Moyen-orientale 0315
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Religion
- Généralités 0318
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- Histoire des religions 0320
- Philosophie de la religion 0322

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SCIENCES SOCIALES
Anthropologie
- Archéologie 0324
- Culturelle 0326
- Physique 0327

Droit 0398
Économie
- Généralités 0501
- Commerce-Affaires 0505
- Économie agricole 0503
- Économie du travail 0510
- Finances 0508
- Histoire 0509
- Théorie 0511

Études américaines 0323
Études canadiennes 0385
Études féministes 0453
Folklore 0358
Géographie 0366
Gérontologie 0351
Gestion des affaires
- Généralités 0310
- Administration 0454
- Banques 0770
- Comptabilité 0272
- Marketing 0338

Histoire
- Histoire générale 0578
- Ancienne 0579
- Médiévale 0581
- Moderne 0582
- Histoire des noirs 0328
- Africaine 0331
- Canadienne 0334
- États-Unis 0337
- Européenne 0335
- Moyen-orientale 0333
- Latino-américaine 0336
- Asie, Australie et Océanie 0332

Histoire des sciences 0585
Loisirs 0814
Planification urbaine et régionale 0999
Science politique
- Généralités 0615
- Administration publique 0617
- Droit et relations internationales 0616

Sociologie
- Généralités 0626
- Aide et bien-âtre social 0630
- Criminologie et établissements pénitentiaires 0627
- Démographie 0938
- Études de l' individu et de la famille 0628
- Études des relations interethniques et des relations raciales 0631
- Structure et développement social 0700
- Théorie et méthodes. 0344
- Travail et relations industrielles 0629

Transports 0709
Travail social 0452

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- Agronomie. 0285
- Alimentation et technologie alimentaire 0359
- Culture 0479
- Élevage et alimentation 0475
- Exploitation des pâturages 0777
- Pathologie animale 0476
- Pathologie végétale 0480
- Physiologie végétale 0817
- Sylviculture et faune 0478
- Technologie du bois 0746

Biologie
- Généralités 0306
- Anatomie 0287
- Biologie (Statistiques) 0308
- Biologie moléculaire 0307
- Botanique 0309
- Cellule 0379
- Écologie 0329
- Entomologie 0353
- Génétique 0369
- Limnologie 0793
- Microbiologie 0410
- Neurologie 0317
- Océanographie 0416
- Physiologie 0433
- Radiation 0821
- Science vétérinaire 0778
- Zoologie 0472

Biophysique
- Généralités 0786
- Medicale 0760

SCIENCES DE LA TERRE
Biogéochimie 0425
Géochimie 0996
Géodésie 0370
Géographie physique 0368
Géologie 0372
Géophysique 0373
Hydrologie 0388
Minéralogie 0411
Océanographie physique 0415
Paléobotanique 0345
Paléoécologie 0426
Paléontologie 0418
Paléozoologie 0985
Palynologie 0427

SCIENCES DE LA SANTÉ ET DE L'ENVIRONNEMENT
Économie domestique 0386
Sciences de l'environnement 0768
Sciences de la santé
- Généralités 0566
- Administration des hôpitaux 0769
- Alimentation et nutrition 0570
- Audiologie 0300
- Chimiothérapie 0992
- Dentisterie 0567
- Développement humain 0758
- Enseignement 0350
- Immunologie 0982
- Loisirs 0575
- Médecine du travail et thérapie 0354
- Médecine et chirurgie 0564
- Obstétrique et gynécologie 0380
- Ophtalmologie 0381
- Orthophonie 0460
- Pathologie 0571
- Pharmacie 0572
- Pharmacologie 0419
- Physiothérapie 0382
- Radiologie 0574
- Santé mentale 0347
- Santé publique 0573
- Soins infirmiers 0569
- Toxicologie 0383

SCIENCES PHYSIQUES
Sciences Pures
Chimie
- Généralités 0485
- Biochimie 487
- Chimie agricole 0749
- Chimie analytique 0486
- Chimie minérale 0488
- Chimie nucléaire 0738
- Chimie organique 0490
- Chimie pharmaceutique 0491
- Physique 0494
- PolymÇres 0495
- Radiation 0754

Mathématiques 0405
Physique
- Généralités 0605
- Acoustique 0986
- Astronomie et astrophysique 0606
- Électronique et électricité 0607
- Fluides et plasma 0759
- Météorologie 0608
- Optique 0752
- Particules (Physique nucléaire) 0798
- Physique atomique 0748
- Physique de l'état solide 0611
- Physique moléculaire 0609
- Physique nucléaire 0610
- Radiation 0756

Statistiques 0463

Sciences Appliqués Et Technologie
Informatique 0984
Ingénierie
- Généralités 0537
- Agricole 0539
- Automobile 0540
- Biomédicale 0541
- Chaleur et thermodynamique 0348
- Conditionnement (Emballage) 0549
- Génie aérospatial 0538
- Génie chimique 0542
- Génie civil 0543
- Génie électronique et électrique 0544
- Génie industriel 0546
- Génie mécanique 0548
- Génie nucléaire 0552
- Ingénierie des systèmes 0790
- Mécanique navale 0547
- Métallurgie 0743
- Science des matériaux 0794
- Technique du pétrole 0765
- Technique minière 0551
- Techniques sanitaires et municipales 0554
- Technologie hydraulique 0545

Mécanique appliquée 0346
Géotechnologie 0428
Matières plastiques (Technologie) 0795
Recherche opérationnelle 0796
Textiles et tissus (Technologie) 0794

PSYCHOLOGIE
Généralités 0621
Personnalité 0625
Psychobiologie 0349
Psychologie clinique 0622
Psychologie du comportement 0384
Psychologie du développement 0620
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Psychologie industrielle 0624
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Psychologie sociale 0451
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "**CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P IN THE BOVINE PARATHYROID GLAND: IMMUNOHISTOCHEMICAL LOCALIZATION AND EFFECT ON PARATHYROID HORMONE SECRETION**" submitted by **Shane T. Mortimer** in partial fulfillment of the requirements for the degree of **Master of Science**.

September, 4, 1992

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ABSTRACT

Bovine parathyroid glands were stained by indirect immunohistochemistry to identify the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP). Nerve fibres containing CGRP- and SP-immunoreactivity were identified throughout the tunica adventitia of arteries and arterioles, where they often made contact with the tunica media. Many of the neuropeptide-immunoreactive nerve fibres deviated from the vasculature and encircled parenchymal lobules. All the immunoreactive nerve fibres were found to contain both CGRP- and SP-immunoreactivity.

Incubating primary bovine parathyroid cell cultures with 10^{-8} M to 10^{-5} M CGRP or SP at normal physiological concentrations (1.25 mM) of ionized calcium (Ca^{++}) resulted in no significant modulation of parathyroid hormone (PTH) secretion for up to 90 min. When CGRP and SP were added together at concentrations between 10^{-10} M and 10^{-6} M, there was no significant effect on PTH secretion for up to 60 min. In the presence of either 0.5 mM or 2.0 mM Ca^{++}, CGRP or SP did not significantly modulate PTH secretion from the cultures for up to 60 min.

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To my parents,

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bPTH	Bovine Parathyroid Hormone
°C	degree Celsius
Ca^{++}	Ionized Calcium
CALC	Calcitonin/Calcitonin Gene-Related Peptide Gene
cAMP	Cyclic 3′,5′-Adenosine Monophosphate
CCK	Cholecystokinin/Gastrin
CGRP	Calcitonin Gene-Related Peptide
CGRP-LI	Calcitonin Gene-Related Peptide-Like Immunoreactivity
cpm	Counts Per Minute
d	day
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
EDTA	Disodium Ethylenediamine Tetraacetate
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
g	gram
Gly	Glycine
h	hour
hCGRP	Human Calcitonin Gene-Related Peptide
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid
^{125}I	Iodine 125
IgG	Immunoglobulin G
i.p.	Intraperitoneal
i.v.	Intravenous
kg	kilogram
l	litre
Leu	Leucine
m	metre
M	molar
Met	Methionine
mg	milligram
Mg^{++}	Ionized Magnesium
min	minute
ml	millilitre
mM	millimolar

mRNA	Messenger Ribonucleic Acid
MTC	Medullary Thyroid Carcinoma
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NKA	Neurokinin A
NKB	Neurokinin B
nm	nanometre
nM	nanomolar
nmol	nanomoles
NPγ	Neuropeptideγ
NPK	Neuropeptide K
NPY	Neuropeptide Y
25-OHD_3	25-Hydroxycholecalciferol
1α,25-$(OH)_2D_3$	1α,25-Dihydroxycholecalciferol
6-OHDA	6-Hydroxydopamine
PB	Phosphate Buffer
PBS	Phosphate-Buffered Saline
PBS-TX	Phosphate-Buffered Saline containing 0.3% Triton X-100
Phe	Phenylalanine
pM	picomolar
pmol	picomoles
PPT	Preprotachykinin
PTH	Parathyroid Hormone
rCGRP	Rat Calcitonin Gene-Related Peptide
RIA	Radioimmunoassay
rpm	Rounds Per Minute
s	second
SD	Standard Deviation
SP	Substance P
SP-LI	Substance P-Like Immunoreactivity
TRITC	Tetramethyl-Rhodamine Isothiocyanate
U	Unit
μl	microlitre
μm	micrometre
μM	micromolar
Val	Valine
VIP	Vasoactive Intestinal Polypeptide

INTRODUCTION

Multicellular organisms have evolved two principal mechanisms to regulate and integrate the function of their different cells: the nervous system and the endocrine system. While the former sends electrochemical signals along axons to target tissues, the latter performs its regulatory function by transporting chemical agents via the bloodstream to affect target tissues. At first, the nervous system and endocrine system appear to be quite separate. However, they are closely interrelated. Many chemical agents have been found to be localized in and secreted by both neuronal presynaptic terminals and endocrine cells. Also, both the nervous and endocrine systems have been shown to modulate the activity of each other. This thesis attempts to add to the growing body of knowledge concerning the effects of the nervous system on endocrine function, especially with regard to the parathyroid gland and calcium homeostasis.

1.1 CALCIUM HOMEOSTASIS

The physiological importance of calcium falls into two broad categories. Approximately 99% of the body's calcium is found in the skeleton (Stewart and Broadus 1987). Calcium in bone exists primarily in the form of small hydroxyapatite crystals composed of calcium, phosphate, and hydroxyl ions, with the formula: $Ca_{10}(PO_4)_6(OH)_2$. The skeleton is designed to carry out the mechanical functions of providing protection for internal organs, is required for

movement by giving rigid support to the extremities and the joints, and serves to transmit the force of muscular contraction from one part of the body to another. Bone also provides a reservoir of calcium, phosphate and other ions essential for a variety of homeostatic functions.

The remaining 1% of the body's calcium is found in serum and extracellular fluids and within cells. Calcium exists in the serum in three fractions. 50% of the calcium is found in an ionized form, 40% is bound to serum proteins (90% to albumins and 10% to globulins), and the remaining 10% is complexed to anions in the blood, mainly bicarbonate, citrate, and phosphate (Marshall 1976; Pedersen 1972). The concentration of calcium in the cytosol is only about one one-thousandth of that found extracellularly, as most of the intracellular calcium is sequestered within the mitochondria and endoplasmic reticulum. Calcium pumps located in the plasma, mitochondrial, and endoplasmic reticular membranes control the concentration of calcium in the cytosol. Calcium leaks passively into the cytosol by diffusion across these three membranes, but these pumps maintain the calcium gradient by actively transporting calcium away from the cytosol. Although only 1% of the calcium is found outside the skeleton, its intracellular compartmentalization and concentration gradient across the cell membrane are essential to the normal functioning of a number of biological processes.

Calcium ions are responsible for linking excitation and contraction in muscle. Skeletal and cardiac muscle utilize the calcium stored within their sarcoplasmic reticulum for contraction. Depolarization of the sarcolemma results in the influx of calcium from the sarcoplasmic reticulum through voltage-gated calcium channels. The abrupt increase in cytosolic calcium binds to troponin C, allowing actin and myosin to form cross-bridges, resulting in contraction of the sarcomere. In smooth muscle, the increase in intracellular calcium results in increased binding of Ca^{++} with calmodulin, triggering myosin light chain kinase to phosphorylate the myosin heads. This allows the actin-myosin interaction, resulting in muscle contraction.

Release of calcium from the endoplasmic reticulum functions as a second messenger system. The binding of a ligand to specific cell receptors activates phospholipase C by a specific G-protein. The active phospholipase C hydrolyzes phosphatidylinositol-4,5-bisphosphate to *myo*-inositol-1,4,5-trisphosphate and diacylglycerol. *Myo*-inositol-1,4,5-trisphosphate releases calcium stores from the endoplasmic reticulum (Berridge 1984). Calcium ions can also bind to and modulate the activities of key enzymes regulating intermediary metabolism (Breslau 1988).

Exocytosis of hormones, neurotransmitters and other cellular products is dependent on a rise in cytosolic calcium in many cells (Knight *et al.* 1989).

Calcium is also an important factor in blood coagulation. Calcium ions are required for promoting all but the first two steps in the blood coagulation cascade (Guyton, 1991a).

Because calcium within the serum and extracellular fluid is so important for the normal functioning of many biological processes, its concentration must be controlled within vary narrow limits. In human beings, serum calcium is maintained between 2.12 and 2.62 mM (Breslau 1988). Any deviation from this narrow range results in pathological conditions.

Responsibility for maintaining calcium within these limits is shared primarily by three hormones: parathyroid hormone, calcitonin, and the active form of vitamin D (1α,25-dihydroxycholecalciferol).

1.1.1 Parathyroid Hormone

Parathyroid hormone (PTH) is the hormone primarily responsible for maintaining calcium homeostasis. PTH is found from amphibians to mammals and is produced by chief cells within the parathyroid gland. PTH is a single-chain linear polypeptide composed of 84 amino acids with a molecular weight of 9500 (Figure 1.1). The PTH gene is located on the short-arm of chromosome 11, and encodes for a larger precursor, termed preproPTH, a polypeptide of 115 amino acids with a molecular weight of 13 000. This precursor is short-lived, and once it is transferred to the endoplasmic reticulum, enzymatic cleavage of the amino-terminal 25 residue leader sequence results in

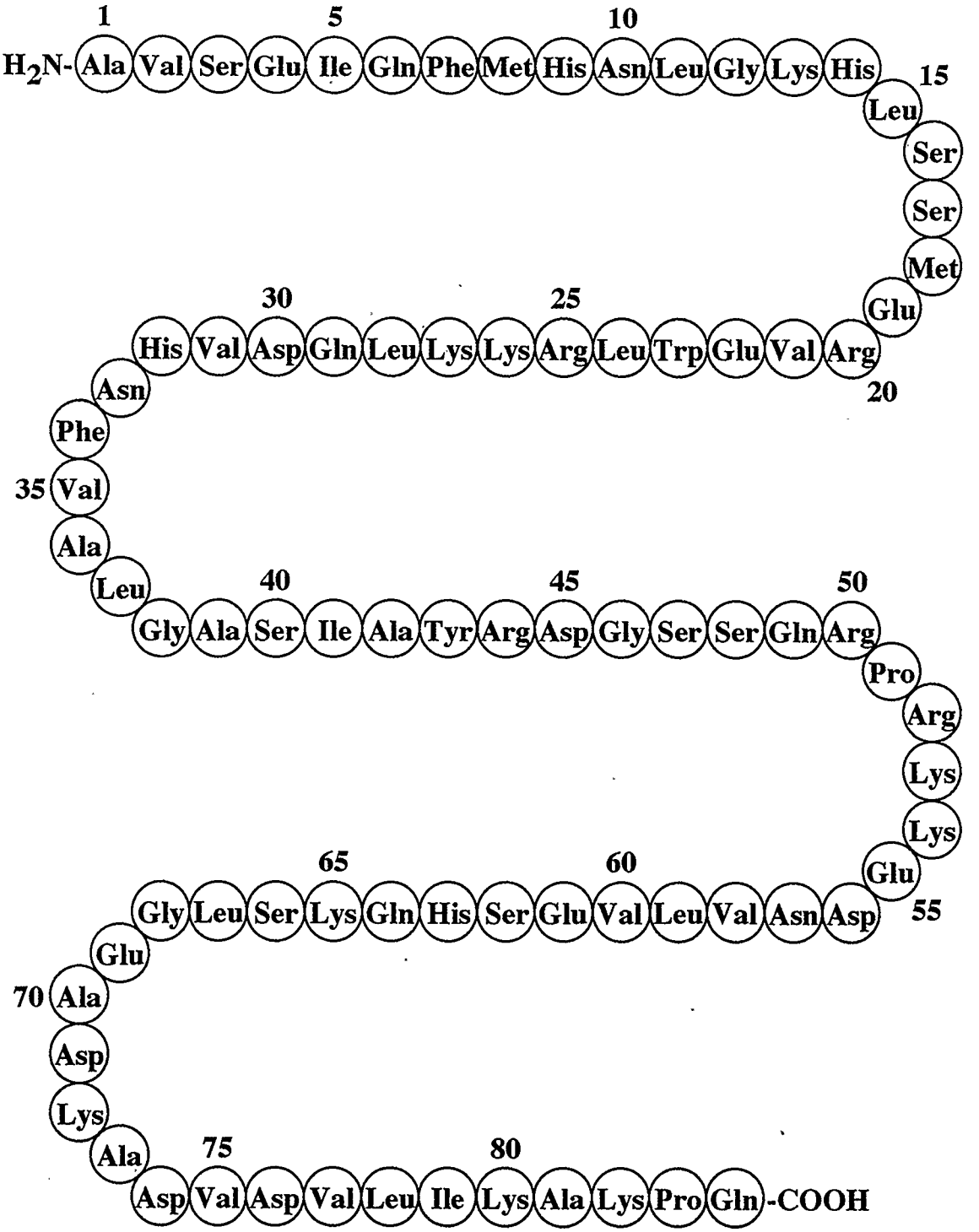


Figure 1.1 The amino acid sequence of bovine parathyroid hormone.

a 90 amino acid proPTH polypeptide with a molecular weight of 10 200. ProPTH is transferred to the Golgi apparatus, where it is cleaved to form the mature PTH molecule which is packaged into secretory granules for storage and subsequent secretion.

The major regulator of PTH secretion is ionized calcium (Ca^{++}) in the serum. The parathyroid chief cell is unusual in that there is an inverse relationship between PTH secretion and serum calcium. Maximum PTH secretion occurs in the presence of low extracellular Ca^{++}, and high concentrations of extracellular Ca^{++} suppress PTH release (Brown 1976; Habener *et al.* 1975; Hanley *et al.* 1980; Mayer and Hurst 1978; Morrissey and Cohn 1978; Targovnik *et al.* 1971). However, these investigators reported that high levels of calcium were unable to completely suppress PTH secretion.

PTH, by interacting with its two main target organs, bone and kidney, increases the concentration of calcium in the extracellular fluid. PTH acts directly on bone by stimulating the combined processes of osteocytic osteolysis and osteoclastic bone resorption to release calcium into the blood. PTH stimulates already existing osteoblasts and osteocytes to absorb bone mineral from bone without resorption or destruction of the bone matrix. This release of calcium from bone in response to PTH occurs rapidly, within minutes, and is termed osteocytic osteolysis (Guyton 1991b). Osteoclastic bone resorption, a second phase of bone resorption, is a delayed response that occurs only after exposure

of bone to prolonged stimulation by PTH. This phase is characterized by the destruction and resorption of bone mineral and bone matrix by osteoclasts, and requires days or weeks to become fully active, involving the recruitment of new osteoclasts (Guyton 1991b). Osteoclasts are highly mobile multinucleated cells, probably derived from extraskeletal monocytic progenitor cells. They move along the bone surface actively resorbing bone. Although PTH increases the activity of osteoclasts, no PTH receptors have been detected on these cells. However, PTH receptors are found on osteoblasts. It is postulated that osteoblasts, under the influence of PTH, stimulate the osteoclasts to resorb bone, but the signal for this is unknown (McSheehy and Chambers 1986).

PTH, by interacting with its receptors in the distal tubule of the nephron, increases the reabsorption of calcium from the tubular filtrate. PTH also decreases the proximal tubular reabsorption of phosphate, causing hypophosphatemia. This results in less phosphate available to complex with calcium, thus increasing the fraction of free Ca^{++} in the serum. In the proximal tubule, in addition to inhibiting phosphate reabsorption, PTH also inhibits reabsorption of sodium and bicarbonate. Binding of calcium to serum proteins increases under alkaline conditions and decreases under acidic conditions (Marshall 1976). The excretion of sodium and bicarbonate stimulated by PTH produces mild diuresis and may provoke a mild hyperchloremic acidosis. This shifts calcium from the bound fraction to the ionized fraction.

As well as interacting with bone and kidney, PTH also increases the absorption of calcium from the intestine. However, the action of PTH is indirect and involves the production of the active form of vitamin D in the kidney (see below).

1.1.2 Calcitonin

Calcitonin, discovered in 1961 by Copp *et al.* (1962), is secreted by C cells of the thyroid gland (Foster *et al.* 1964). The C cells are derived from neural crest cells (Pearce and Polak 1971). Calcitonin is a 32 amino acid peptide with a 1-7 disulphide bond and a carboxy-terminal proline amide residue. The main stimulus for the secretion of calcitonin is an elevated serum calcium concentration (Heynen and Franchimont 1974; Parthemore *et al.* 1975; Parthemore and Deftos 1978), although certain gastrointestinal hormones are also secretagogues (Care *et al.* 1971; Heath and Sizemore 1977; Parthemore and Deftos 1978).

Calcitonin protects against hypercalcemia, and thus antagonizes the action of PTH. The main mechanism of action of calcitonin is to inhibit the release of calcium from bone through its actions on osteoclasts, which contain 10^6 calcitonin receptors per cell (Nicholson *et al.* 1986). Activation of these receptors results in a reduction in osteoclast motility and spreading, with a loss of their ruffled borders, which indicates a decline in bone resorption (Chambers *et al.* 1986; Chambers and Magnus 1982; Chambers and Moore 1983). It has also been shown that calcitonin directly inhibits resorption of cortical bone by isolated

osteoclasts (Chambers *et al.* 1984, 1985). However, the importance of calcitonin in calcium homeostasis has been questioned, as there are no clinical manifestations of either an overproduction or underproduction of calcitonin. Calcitonin may have a physiological role in protecting the skeleton during times of stress, such as during childhood, pregnancy, and lactation.

1.1.3 Vitamin D_3

Vitamin D_3 and its metabolites are steroid hormones; their metabolism and mechanism of action have much in common with those of other steroid hormones. The ultraviolet irradiation of the skin causes 7-dehydrocholesterol to be converted into previtamin D_3. Overproduction of previtamin D_3 is prevented by the photochemical equilibrium that favours the production of the inert metabolites lumisterol and tachysterol during periods of prolonged sun exposure. Over a period of several days, the previtamin D_3 undergoes a temperature-dependent isomerization to vitamin D_3 (cholecalciferol). The vitamin D-binding protein in serum has a 1000-fold higher affinity for cholecalciferol than for previtamin D_3, so that cholecalciferol is transported preferentially into the circulation. Cholecalciferol is a biologically inactive prohormone. The first step in the activation pathway involves enzymatic 25-hydroxylation of cholecalciferol in the liver to form 25-hydroxycholecalciferol (25-OHD_3). This conversion is not tightly regulated, and 25-OHD_3 constitutes the major circulating form of vitamin D in humans. 25-OHD_3 is transported to the kidney where it is hydroxylated at

C-1 to produce 1α,25-dihydroxycholecalciferol (1α,25-$(OH)_2D_3$), the active metabolite of vitamin D_3. The 1α-hydroxylation of 25-OHD_3 is tightly regulated and constitutes the rate-limiting step in the production of 1α,25-$(OH)_2D_3$. PTH is the principal activator of the renal synthesis of 1α,25-$(OH)_2D_3$. 1α,25-$(OH)_2D_3$ acts on the intestinal epithelial cells by increasing the transcription and production of calbindin-D_{9k} (Gross and Kumar 1990), which facilitates calcium transport across the luminal surface of the duodenum.

1.2 THE PARATHYROID GLAND

1.2.1 Embryology and Histology

The parathyroid glands are derived from the endodermal germ layer of the third and fourth pairs of branchial pouches (Moore 1988). The superior parathyroid glands are derived from the fourth branchial pouches. In cattle, they remain almost stationary during embryologic development, accounting for their final location medial to the common carotid artery just proximal to its bifurcation into the external and internal carotid arteries (Figure 1.2).

The inferior parathyroid glands develop from the third branchial pouches. In cattle, the inferior parathyroid glands migrate caudally with the thymus until they separate from it, assuming their final position partially embedded in the medial surface of the thyroid gland near the trachea (Roth and Schiller 1976) (Figure 1.2).

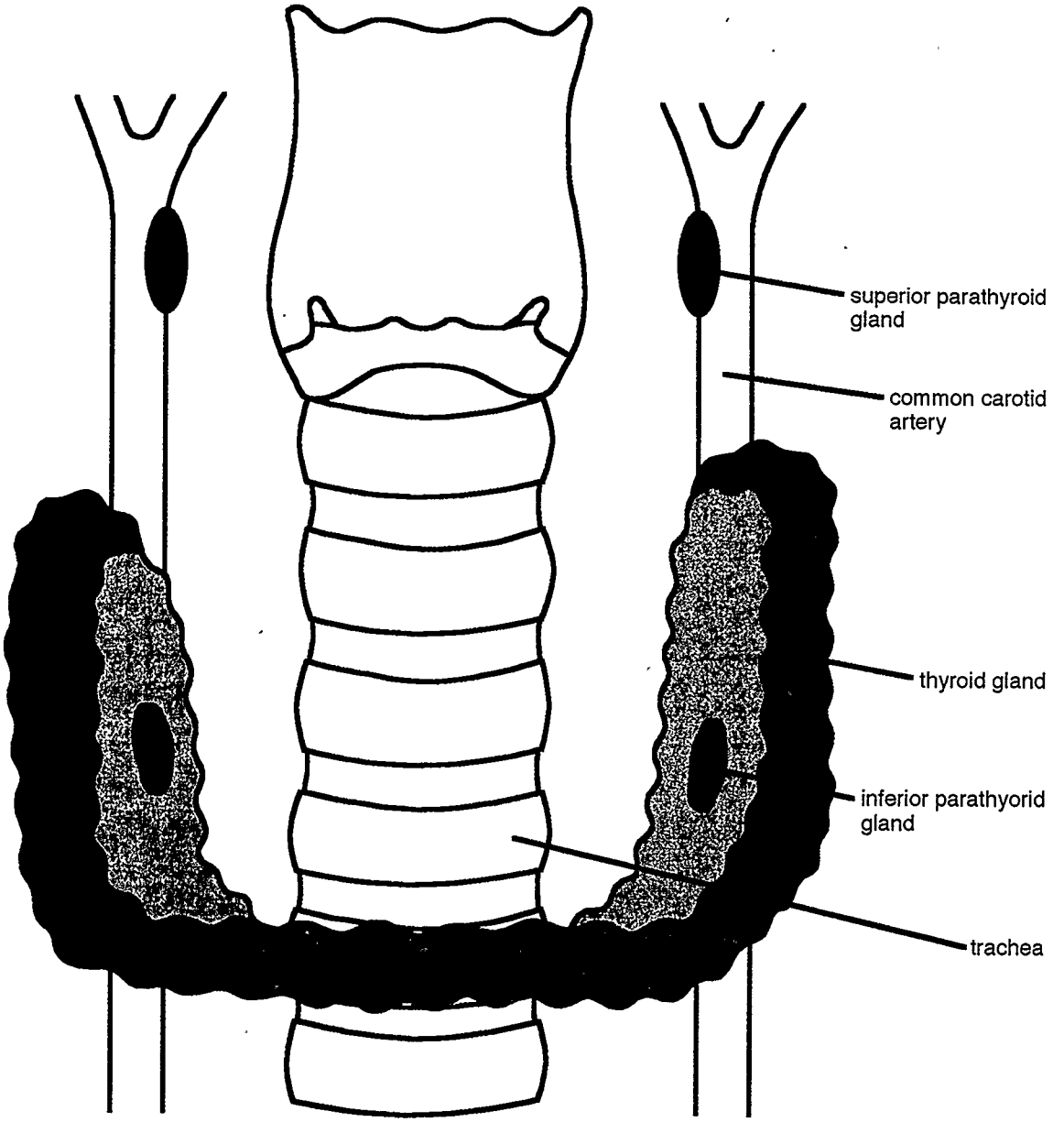


Figure 1.2 Location of the parathyroid glands in the bovine neck. The superior parathyroid glands are situated proximal to the bifurcation of the common carotid arteries, while the inferior parathyroid glands are imbedded within the thyroid gland.

The principal cell within the parathyroid parenchyma is the chief cell, which is responsible for the synthesis and secretion of PTH. The chief cell has the typical appearance of an active secretory cell. It has a prominent Golgi apparatus and rough endoplasmic reticulum, as well as many secretory granules. Chief cells in the bovine parathyroid gland are arranged in cords and sheets (Capen *et al.* 1965).

Levine (1928) also identified oxyphil cells in the bovine parathyroid gland. The cells were few in number and usually scattered as single cells among the chief cells. The oxyphil cells contain a poorly developed endoplasmic reticulum and Golgi apparatus, and therefore appear not to be normally active secretory cells. Oxyphil cells are absent in many species including young human beings. Their precise function to date is still unknown, although they are usually packed with mitochondria and contain higher levels of energy-producing enzymes than the chief cells (Tremblay and Carter 1961).

1.2.2 Innervation

Rhinehart (1912) gave the first account of the innervation of the parathyroid glands. He found perivascular nerve plexuses in the arteries of the gland. The branching of the nerves accompanied the branching of the arteries, so that each smaller artery carried a single nerve fibre. No nerves were found around veins or capillaries. Since all the nerves ended in the vessel walls, Rhinehart was of the opinion that the nerves were restricted to a vasomotor function. Since then,

several authors have reported unmyelinated nerve fibres to be located primarily within perivascular spaces in the parathyroid gland of several species, including the bovine (Capen *et al.* 1965; Jacobowitz and Brown 1980; Mazzocchi *et al.* 1967; Munger and Roth 1963; Roth and Munger 1962; Unsicker 1971; Yeghiayan *et al.* 1972; Zawistowski 1966).

Raybuck (1952) found the perivascular plexuses in dogs and cats to be composed of two morphologically distinct fibre types. He identified large myelinated fibres located within the superficial portion of the tunica adventitia and smaller unmyelinated fibres located adjacent to the tunica media, to which he assigned a postganglionic sympathetic vasomotor role. Raybuck noted that some of the unmyelinated fibres deviated from the arterial walls and entered the parenchyma of the gland where they terminated in dark knob-like swellings in intimate relationship with the chief cells. In some instances the individual fibres were interlaced with other fibres, giving the appearance of a plexus among the chief cells. In other instances, nerve fibres appeared to enter directly into the cytoplasm of the chief cells. Using electron microscopy, Altenähr (1971) frequently found neuroepithelial synapses between axons and chief cells in human beings, with synaptic clefts measuring 15 nm. The preterminal axons were also rich in neurosecretory granules. Nerve fibres ending in dark knob-like swellings in intimate relationship with chief cells, observed by light microscopy, electron microscopy, and fluorescence histochemistry, have also

been reported by others (Atwal 1981; Mikhail 1971; Norberg *et al.* 1975; Wideman 1980).

Some investigators have been able to trace the unmyelinated fibres to their origin within the cervical chain ganglia, supporting the assumption of earlier investigators that these fibres are sympathetic. The origin of the fibres appears to show some species specificity. In the rat, the parathyroid glands are innervated by postganglionic perikarya located in the medial and/or inferior cervical ganglia, which send their axons through the superior cervical ganglion to reach the glands via the external carotid nerve (Romeo *et al.* 1986). There are no perikarya in the superior cervical ganglion of the rat that send axons to the thyroid or parathyroid glands. However, in the rabbit, Shoumura *et al.* (1983) found numerous labelled neurons in the superior cervical ganglion, but not the inferior cervical ganglion following horseradish peroxidase injection into the parathyroid gland. In the cat and dog, removal of the cervical portion of the sympathetic trunk resulted in a complete degeneration of unmyelinated fibres from the vasculature that coursed among the chief cells (Raybuck 1952). Using fluorescence histochemistry, Jacobowitz and Brown (1980) were able to confirm the presence of adrenergic nerve terminals on the vasculature in the bovine parathyroid gland, although their distribution was sparse.

Mikhail (1971), using light microscopy, reported the presence of parasympathetic terminal ganglia scattered throughout the parathyroid gland in

dogs, guinea-pigs, and rabbits. The dendrites extended between the neighbouring chief cells, forming a delicate plexus between the cells. These findings are supported by Shoumura *et al.* (1983), who found acetyl-cholinesterase positive nerve fibres in the rabbit parathyroid gland, and labelled cell bodies in the dorsal motor nucleus of the vagus after horseradish peroxidase injection into the parathyroid gland.

Raybuck (1952) believed that the large myelinated fibres located within the superficial portion of the tunica adventitia were sensory components of the vagus nerve. Atwal (1981), who identified myelinated fibres in the interstitium of the canine parathyroid gland, also suggested these fibres were afferent as they were independent of the walls of blood vessels and had an average diameter of 7.04 μm. Myelinated presynaptic fibres characteristically have diameters of 3 μm or less (Pick 1970). Wideman (1980) reported vagal fibres near vascular smooth muscle, as well as adjacent to the chief cells, in the parathyroid glands of the European starling. Most of the myelinated fibres showed signs of degeneration ten days after nodose ganglionectomy. However, some fibres retained a normal ultrastructural appearance, suggesting additional sources for the myelinated fibres. It is conceivable that they are preganglionic parasympathetic fibres.

1.3 NEURAL CONTROL OF PARATHYROID HORMONE SECRETION

While extracellular Ca^{++} is the principal factor regulating PTH release, the presence of nerves within the parathyroid gland suggests the nervous system might directly influence chief cell activity. Previous studies have demonstrated that adrenergic agonists can modulate the secretion of PTH. Beta-adrenergic agonists bind to specific receptors on dispersed parathyroid cells (Brown *et al.* 1977c). Norepinephrine, epinephrine, and dopamine cause an increase in cyclic 3′,5′-adenosine monophosphate (cAMP) production by parathyroid cells, and a dose-dependent increase in PTH secretion *in vitro* and *in vivo* (Brown *et al.* 1976, 1977a, 1977b, 1977c, 1978b, 1983; Fischer *et al.* 1973; Hanley *et al.* 1980; Hanley and Wellings 1985). The response to norepinephrine, epinephrine, and dopamine can be augmented by dibutyryl cAMP, isobutylmethoxamine, or theophylline (Abe and Sherwood 1972; Brown *et al.* 1977b, 1978a) and blocked by catecholamine antagonists (Brown *et al.* 1976, 1977a, 1977b, 1977c, 1978a, 1978b; Hanley *et al.* 1980). Brown *et al.* (1978b) reported that selective α-adrenergic activation decreases catecholamine-stimulated cAMP production and PTH secretion from dispersed bovine parathyroid cells. Isoproterenol, a pure β-agonist, is more potent than norepinephrine and epinephrine. This may be due to α-adrenergic activation by norepinephrine and epinephrine. Blum *et al.* (1978), Kukreja *et al.* (1975), and Metz *et al.* (1978) were unable to show any effect of α-adrenergic agonists on PTH secretion *in vivo*.

MacGregor *et al.* (1973) identified two pools of PTH in the parathyroid gland. One population contained newly synthesized PTH, and the other contained an older storage pool of PTH. Work by Morrissey and Cohn (1979) and Hanley *et al.* (1980) demonstrated that activation of cAMP caused secretion from the storage pool, but had no effect on the newly synthesized pool. As only the storage pool of PTH is responsive to catecholamines, these agents are only able to cause a transient release of PTH, lasting for several minutes. While subsequent stimulation by catecholamines causes a dose-dependent increase in cAMP, no further PTH is secreted by the chief cells. It is therefore believed that the cells have depleted their storage pools of PTH. Nonetheless, the chief cells are still able to secrete PTH in response to low calcium, which induces the release of newly synthesized PTH, which is continually being replenished. Catecholamines have their greatest effect on stimulating PTH secretion under hypocalcemic conditions. The effect of catecholamines is diminished under normal calcium conditions, and they are unable to stimulate PTH secretion during a state of hypercalcemia. This may protect against the release of PTH by catecholamines during times when there is no need for more extracellular calcium.

The primary question still not answered is what physiological role the nervous system, its neurotransmitters, and circulating catecholamines have in parathyroid gland function. Vora *et al.* (1980) reported that electrical stimulation of the

superior cervical ganglion resulted in a 30% increase in PTH secretion in the rat. In the dog, electrical and chemical stimulation of the cervical vagosympathetic trunk failed to affect PTH release (Heath *et al.* 1985).

The increase in PTH secretion caused by disodium ethylenediamine tetra-acetate (EDTA) induced hypocalcemia, is blunted after either adrenalectomy (to remove the primary source of epinephrine), or chemical sympathectomy with 6-hydroxydopamine (6-OHDA) in rats (Vora *et al.* 1978). However, Heath *et al.* (1980) reported no change in PTH levels over controls in response to a hypocalcemic challenge using the same treatments. Cardinali and Ladizesky (1985) found that hypocalcemia induced by intraperitoneal (i.p.) administration of 100 mg/kg body weight EDTA every 30 min resulted in a much greater decrease in serum calcium levels in the superior cervical ganglionectomized rats than in sham-operated control rats. They also found that the elevation in PTH levels caused by EDTA was considerably higher in control rats than in the ganglionectomized animals. However, Heath *et al.* (1980) demonstrated that hypocalcemia caused no difference in PTH levels between controls and rats chemically sympathectomized with 6-OHDA.

Morii *et al.* (1963) found that vagotomy resulted in an accelerated recovery of total serum calcium to induced hypocalcemia in dogs, which was mimicked by atropine. Isono and Shoumura (1980) found a proliferation of the Golgi apparatus, and an increase in ribosomes and secretory granules in rabbits 24 h

after vagotomy. This suggests an inhibitory role of the vagus on PTH secretion, with a stimulation of the synthesis and release of PTH in the vagotomized rabbit. Williams *et al.* (1985) demonstrated an inhibition of PTH secretion by cholinergic agonists *in vitro* and *in vivo*, which were blocked by atropine.

The presence of adrenergic nerves terminating on chief cells in certain species, together with the known effects of some catecholamines on PTH secretion, suggests that these fibres may play an important role in the secretory activity of the chief cells. Although neural activity appears to have little effect on basal PTH output when the concentration of Ca^{++} is normal, sympathetic nerves might play a role in PTH secretion under hypocalcemic conditions by transiently stimulating the release of a storage pool of PTH.

There is also evidence that serum PTH and calcium levels undergo circadian or pulsatile variations. Jubiz *et al.* (1972) demonstrated that human PTH levels remain constant throughout the daytime, but start to rise at 8:00 p.m., progressively increasing until a maximum level is attained between 2:00 a.m. and 4:00 a.m. Serum PTH returns to its initial value by 8:00 a.m. Sinha *et al.* (1975) found serum PTH levels highest in man between 8:00 a.m. and 2:00 p.m, while Arnaud *et al.* (1971) found the levels progressively increased from 12:00 noon to 8:00 p.m. In contrast, Kripke *et al.* (1978) and Parthemore *et al.* (1978) found several distinct increases in PTH concentration during the night. Kripke *et al.* (1978) found the peaks tended to recur about every 100 min, and were closely

related to the sleep stages. In addition, there were no clear relations between the circadian or pulsatile variations in serum PTH levels and plasma calcium levels. Fox *et al.* (1981) found oscillations in serum PTH levels in dogs with a period of 12 min which were disrupted by hypocalcemia. These studies suggest that the parathyroid glands are under some regulatory influence from the central nervous system.

1.4 INTRODUCTION TO THE NEUROPEPTIDES

It was once believed that neurons secreted only small molecule transmitters, such as acetylcholine, monoamines, and the amino acids glycine, glutamine, glutamate, and γ-aminobutyric acid. The first realization that neurons could secrete peptides came from the discovery that the hormones, oxytocin and vasopressin, which are secreted from neurons in the posterior hypophysis, were found to be nonapeptides. Later, the peptides adrenocorticotropin releasing hormone, somatostatin, and thyrotropin-releasing hormone, were also found to be secreted from nerves originating in the hypothalamus. These findings were the first to indicate that neurons could in fact secrete peptides. However, it was believed that these hypothalamic-hypophyseal axis neurons were unique. Since then, several other hormones, including adrenocorticotropin, glucagon, insulin, and prolactin have also been found to be secreted from neurons. The gut hormones cholecystokinin, gastrin, secretin, and vasoactive intestinal polypeptide,

were also localized within the nervous system by immunohistochemistry. Several other peptides, which at present appear to be unique to the nervous system, have been found. The number of known new peptides secreted from neurons, called neuropeptides, is steadily increasing (Hökfelt *et al.* 1980).

Several characteristics of neuropeptides distinguish them from the small-molecule neurotransmitters. Neuropeptides are formed like all other proteins destined for secretion. The DNA sequence for a specific neuropeptide is transcribed into mRNA, then translated into a peptide sequence on ribosomes, which is then transported into the endoplasmic reticulum. The peptide is further processed within the Golgi apparatus and then packaged into neurosecretory granules. In contrast, the classical neurotransmitters (biogenic monoamines, acetylcholine and basic amino acids) are synthesized in axon terminals, where they are released. However, secretory granules containing the neuropeptides must be transported from the perikaryon, the length of the axon, to their terminals where the neuropeptides are released. Because of the laborious method of forming the neuropeptides, much smaller quantities of these are usually released than for the small-molecule transmitters. However, this is partially compensated for by the fact that the neuropeptides are generally a thousand or more times potent than the small-molecule transmitters (Guyton 1991d). Removal of the small-molecule transmitters occurs within milliseconds by diffusion away from the synaptic cleft, destruction by enzymes

located within the cleft, and by reuptake of the transmitter by the presynaptic terminals. The neuropeptides appear to be removed by destruction within a few minutes to several hours by specific or nonspecific proteolytic enzymes. Therefore, neuropeptides usually cause a much more prolonged effect.

1.5 SUBSTANCE P

1.5.1 Discovery

The first neuropeptide discovered was substance P (SP). von Euler and Gaddum (1931) found acid alcohol extracts of equine brain and intestine caused a slow contraction of isolated rabbit's intestine and lowered arterial blood pressure. The effects were not blocked by atropine, thus ruling out choline esters as the active agent. They were also able to separate the substance's hypotensive effects from those of adenosine, for unlike adenosine it was unstable in alkali. The structure of SP was not determined until 40 years after its discovery (Chang *et al.* 1971).

1.5.2 Tachykinin Family

SP has been identified as an undecapeptide, belonging to a class of structurally related bioactive neuropeptides called the tachykinins. This family of peptides share a consensus aminated C-terminal sequence; -Phe-Xxx-Gly-Leu-Met-NH_2, where the Xxx residue is either Phe or Val (Figure 1.3). Tachykinins are found throughout the animal kingdom; being identified from cephalopods to

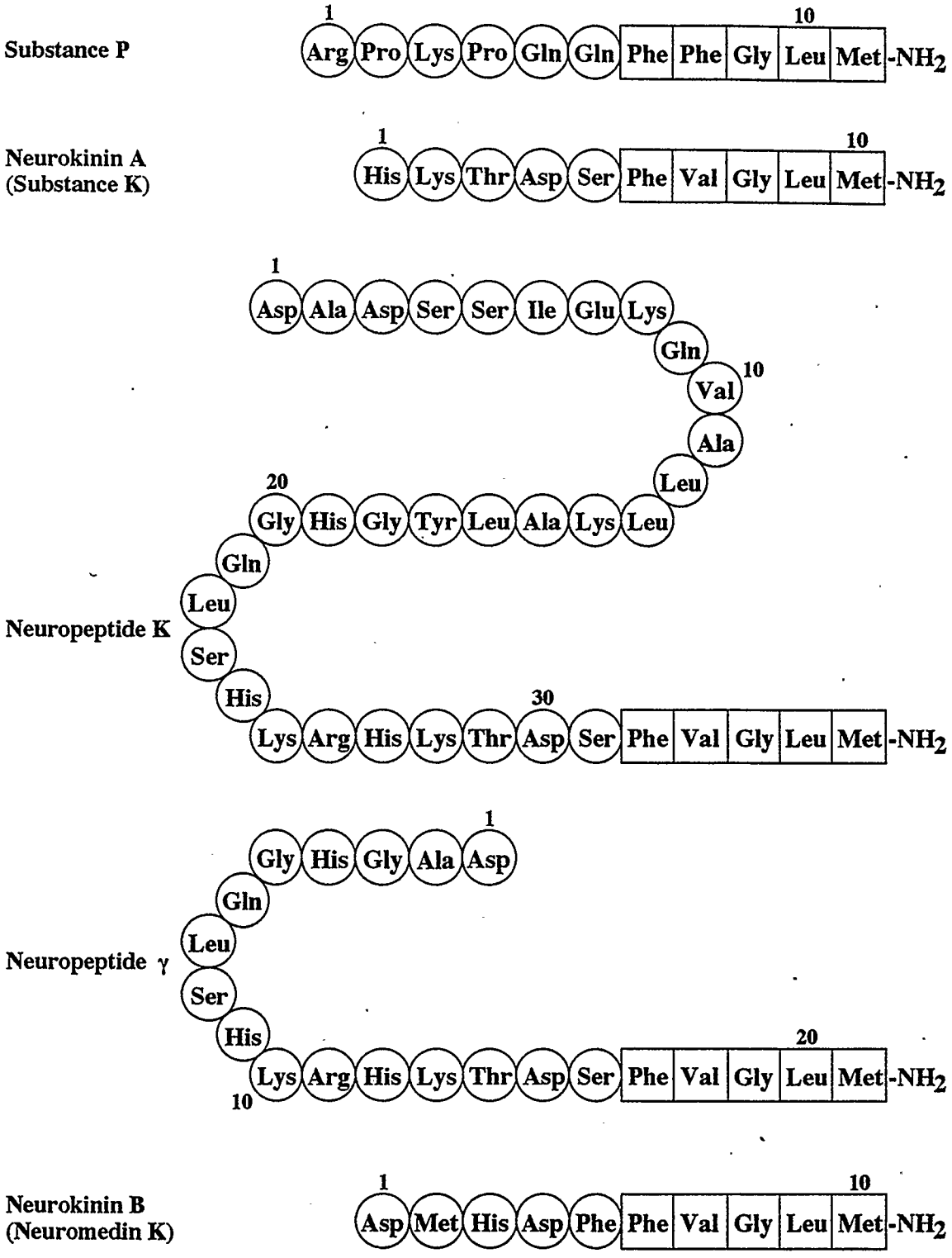


Figure 1.3 Amino acid sequences of the mammalian tachykinins. Amino acids depicted by squares represent the tachykinin consenses sequence.

mammals. For many years SP was considered to be the only tachykinin present in mammals. In 1983, two unique tachykinins were identified in mammals; neurokinin A (NKA), and neurokinin B (NKB) (Kangawa *et al.* 1983; Nawa *et al.* 1983). More recently, two N-terminally extended derivatives of NKA, neuropeptideγ (NPγ), and neuropeptide K (NPK), have also been identified (Kage *et al.* 1988; Tatemoto *et al.* 1985) (Figure 1.3).

SP, NKA, NPγ, and NPK are derived from the first preprotachykinin (PPT) gene (PPT I) to have been isolated. Three different SP-encoding mRNAs are produced from the PPT I gene as a consequence of differential splicing in which the 6th exon sequence is excluded from α-PPT mRNA, the 7 exon sequences are present in β-PPT, while the 4th exon sequence is excluded from γ-PPT mRNA. SP is encoded in part of the 3rd exon, whereas NKA is encoded in part of exon 6. These differentially spliced SP-encoding mRNAs differ in their protein coding sequences, and thus have the ability to encode different peptide products. Different peptides can be produced from the NKA portion of β- and γ-PPT precursors. Thus, either NKA and/or NPK can be produced from β-PPT, and either NKA and/or NPγ can be produced from γ-PPT (Krause *et al.* 1990).

The mammalian tachykinin peptide NKB is produced from a distinct PPT II gene, and is the only known peptide derived from this gene.

1.5.3 Distribution and Biological Activity

Fast-sharp pain is transmitted in peripheral nerves to the spinal cord by small myelinated type Aδ afferent fibres at velocities of 5-30 m/s. Slow-chronic pain is transmitted at velocities of 0.5-2 m/s by very-small unmyelinated type C fibres (Shepherd 1988). On entering the spinal cord from the dorsal spinal roots, the pain fibres either ascend or descend one to three segments in the tract of Lissauer that lies immediately posterior to the dorsal horn of the spinal cord grey matter. The C fibres terminate in laminas II and III (substantia gelatinosa) of the dorsal horns. The signals pass through one or more additional neurons within the dorsal horn before entering mainly lamina V, also within the dorsal horn. The last neuron in the pathway transmits the signal in the ipsilateral spinothalamic tract to the brain stem and thalamus. SP is believed to be the synaptic transmitter released by the C fibres in the substantia gelatinosa. SP is slow to build up at the synapse and also slow to be destroyed. Therefore, the concentration of SP at the synapse is believed to increase for at least several seconds, and perhaps much longer, after pain stimulation begins (Guyton 1991c).

Bayliss (1901) demonstrated that antidromic stimulation of the peripheral stump of transected dorsal roots or sensory nerves induced vasodilation in the canine skin. More recently, it has been realized that up to 90% of the SP synthesized in the cell body of C fibres is transported to the peripheral dendritic

terminals (Brimijoin *et al.* 1980). Here SP is released from the peripheral terminals by noxious stimuli and local tissue inflammation. This has led to the concept of an axon reflex, where sensory nerve fibres bifurcate in the periphery, one branch forming the sensory ending for reception of an irritant stimulus, the other supplying blood vessels and mast cells. When the sensory ending is activated, nerve impulses travel not only centrally to the spinal cord, but also pass antidromically at the other branch points which terminate on the blood vessels. This results in the observed vasodilation in the vicinity of the noxious stimulus.

A great deal of evidence suggests that the release of SP, and perhaps other neuropeptides, by an axon reflex in response to noxious stimuli, can induce local inflammation. Acute inflammation elicited by substances released from sensory nerve fibres is termed neurogenic inflammation (Payan *et al.* 1984). SP causes an increase in tumoricidal and antimicrobial activity of macrophages (Peck 1987), stimulates phagocytosis by macrophages and polymorphonuclear leukocytes (Bar-Shavit *et al.* 1980), promotes monocyte and neutrophil chemotaxis (Marasco *et al.* 1981; Ruff *et al.* 1985), and evokes lysosomal enzyme release from neutrophils (Marasco *et al.* 1981).

Release of SP from sensory fibres of the trigeminal nerve within the eye causes miosis and increases intraocular pressure and protein extravasation into the eye. SP-LI fibres are found in the respiratory and urogenital tracts.

Activation of afferent fibres causes an increase in blood flow and vascular permeability within the respiratory mucosa and urogenital epithelium. Sensory nerve mechanisms also appear to contribute to the development of inflammation in the joints, which may be mediated in part by SP (Holzer 1988).

The gastrointestinal tract is also innervated by extrinsic SP-IR axons, which reach the intestine via the mesenteric nerves and most likely represent sensory nerve fibres passing through the prevertebral sympathetic ganglia. Furthermore, SP-IR fibres in the vagus terminate in the stomach and the intestine. SP-IR neurons in the myenteric ganglia supply the circular muscle, the submucosa, and the mucosa, while the submucosal ganglia only supply the mucosa (Holzer 1988). It is likely that the sensory neurons in the gastrointestinal tract are involved in defence mechanisms, and protect the mucosa against ulceration (Holzer and Sametz 1986).

A great deal of work has gone into trying to identify the mammalian tachykinin receptors. However, the work has been hampered by the slow development of highly specific agonists and antagonists, and the receptor heterogeneity found in many tissues. To date, three receptor types, NK_1, NK_2, and NK_3 have been characterized (Regoli *et al.* 1988). The NK_1 receptors are found on the endothelium and have a rank order of potency: SP > NKA > NKB. Activation by tachykinins may cause vasodilation through the endothelial release of endothelium-derived relaxing factor(s) (Minami *et al.* 1989). NK_2 receptors are

found on rabbit pulmonary artery smooth muscle, where activation causes a dose dependent vasoconstriction. The rank order of potencies for the NK_2 receptors are: NKA > NKB > SP. NK_3 receptors in peripheral vessels are probably involved in vasodilation or protein extravasation, and have a rank order of potency: NKB > NKA > SP.

1.6 CALCITONIN GENE-RELATED PEPTIDE

1.6.1 Discovery

Medullary thyroid carcinoma (MTC), a tumor of the thyroid C cells, is usually associated with elevated calcitonin secretion (Foster 1968). Rosenfeld *et al.* (1981) found that serial transplantations of MTC in rats resulted in a spontaneous and permanent decrease in calcitonin biosynthesis by more than ten-fold. However, they reported that the reduction in calcitonin was associated with a disappearance of the normal form of calcitonin mRNA and its replacement by a slightly larger form. They correctly postulated that alternate processing of the calcitonin gene was occurring.

1.6.2 Peptide Sequence and Gene Structure

In 1982, Amara *et al.* (1982) identified the predicted peptide from the alternately processed mRNA from rat MTC. They identified the peptide as calcitonin gene-related peptide (CGRP), a 37 amino acid peptide in rat which contained a 2-7 disulphide bond and terminated in a phenylalanine-amide

(Figure 1.4). Morris *et al* (1984) identified CGRP from human MTC, which differed from rat CGRP by four residues (positions 1, 3, 25 and 35). A second CGRP, termed βCGRP to distinguish it from the previously discovered αCGRP, has also been identified (Amara *et al.* 1985; Steenbergh *et al.* 1985). In man and rat, βCGRP differs from the α-sequence by three and one amino acids, respectively (Figure 1.4). CGRP has a 30% homology with salmon calcitonin (Breimer *et al.* 1988).

The gene which encodes for both calcitonin and CGRP (CALC) consists of six exons. The first three exons are common to both calcitonin and CGRP mRNA. However, the first exon is not translated. The fourth exon contains the sequences for calcitonin and its C-terminal flanking peptide, katacalcin. The fifth exon contains the CGRP sequence. The sixth exon is also part of the CGRP transcript, but is not translated. Both calcitonin and CGRP mRNA transcripts contain a common amino-terminal flanking peptide with the first 75 amino acids being identical (Gkonos *et al.* 1986).

The CALC-I gene, which encodes αCGRP and calcitonin, is located on the short arm of chromosome 11, between the catalase and PTH genes (Höppener *et al.* 1984; Przepiorka *et al.* 1984). A second calcitonin/CGRP gene, CALC-II, has also been identified on the short arm of chromosome 11 in human and rat (Amara *et al.* 1985; Höppener *et al.* 1985; Steenbergh *et al.* 1985). While the

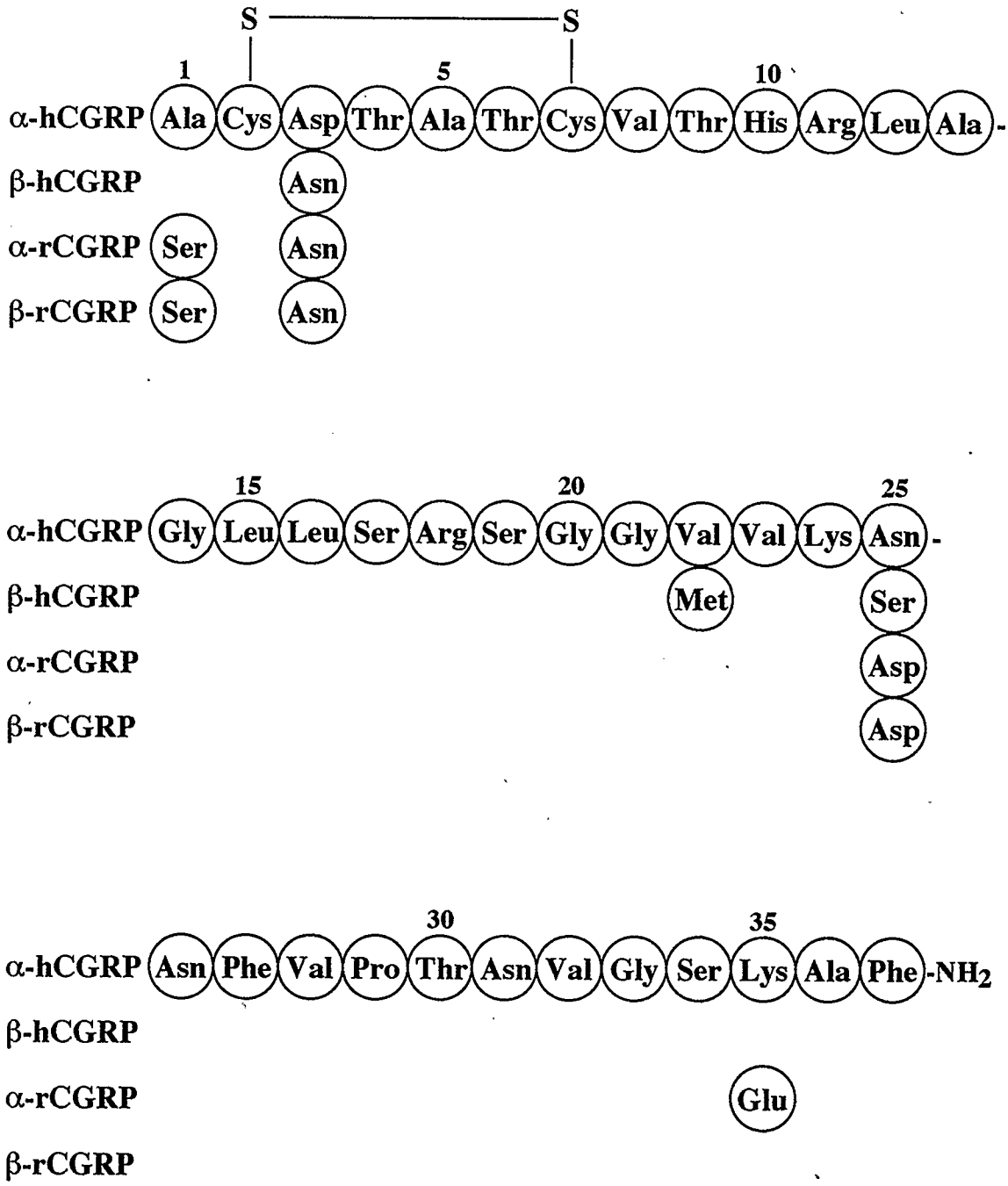


Figure 1.4 Comparison of the α and β amino acid sequences of human and rat calcitonin gene-related peptides.

CALC-II gene encodes βCGRP, it has no known functional calcitonin sequence (Alevizaki *et al.* 1986).

1.6.3 Distribution and Biological Activity

CGRP has been identified in the pituitary, thyroid, and in the central and peripheral nervous systems (Rosenfeld *et al.* 1983). However, there are no reports of calcitonin being produced in neural tissue. The mechanism for the alternate processing of calcitonin and CGRP is still not known. In the brain, the distribution of αCGRP and βCGRP mRNA are similar, but in each region, βCGRP mRNA expression is less than 20% that of αCGRP mRNA (Amara *et al.* 1985). The mRNA levels for βCGRP in the thyroid is also less than 20% that of αCGRP. However, in the nuclei of the third, fourth, and fifth cranial nerves, mRNA levels for βCGRP exceeds that of αCGRP (Amara *et al.* 1985). No functional roles for the differential expression of αCGRP and βCGRP have been established.

CGRP appears to be produced in the normal C cells in man, but at 1/95th that of calcitonin. CGRP has been found in the circulation of man, with normal values reported to range from 0.25 pM to over 250 pM. Girgis *et al.* (1985) found circulating levels of CGRP five times that of calcitonin, with a mean concentration of 25 ± 1.2 pM. The circulating levels of CGRP originate predominantly from its release from nerve terminals (Bevis *et al.* 1986; Emson and Zaidi 1989; Zaidi *et al.* 1985, 1986).

Immunoreactive CGRP has a widespread distribution throughout the cardiovascular system. The intravenous (i.v.) injection of CGRP results in tachycardia accompanied by peripheral vasodilation (Fisher *et al.* 1983; Struthers *et al.* 1985). CGRP is one of the most potent vasodilators known, being more potent than acetylcholine, adenosine triphosphate, histamine, or prostaglandins E_2 and I_2 on the arterial vasculature (Brain *et al.* 1985, 1986a, 1986b). In the heart, CGRP has a positive inotropic and chronotropic effect on contractility (Holman *et al.* 1986; Sigrist *et al.* 1986; Tippins *et al.* 1984). This effect is not mediated via norepinephrine, histamine, or prostaglandins. It is possible that CGRP released locally from cardiac nerves binds to specific receptors to modulate cardiac contractility (Sigrist *et al.* 1986).

CGRP is found co-localized with SP in the dorsal root ganglia, dorsal motor horns, and sensory nerve terminals (Franco-Cereceda *et al.* 1987; Gibson *et al.* 1984; Ju *et al.* 1987; Lee *et al.* 1985a, 1985b; Skofitsch and Jacobowitz 1985; Wiesenfeld-Hallin *et al.* 1984). There is direct evidence that CGRP is released along with tachykinins from sensory nerve endings (Diez Guerra *et al.* 1988). CGRP also potentiates the release of SP from primary sensory terminals (Oku *et al.* 1987). It is likely that CGRP, along with the tachykinins, are important in neurogenic inflammation. Gamse and Saria (1985) have shown that CGRP potentiates the effects of SP, NKA, and NKB, when they are co-injected into rat skin. This mode of action of CGRP appears to be the prevention of SP

degradation by peptidases (Le Grevès *et al.* 1985).

CGRP has been localized to α-motoneurons in the ventral spinal cord (Franco-Cereceda *et al.* 1987; Gibson *et al.* 1984; Rosenfeld *et al.* 1983), and to motor nuclei of cranial nerves (Takami *et al.* 1985). CGRP has also been localized to secretory vesicles in axon terminals at neuromuscular synapses (Takami *et al.* 1985) and has been demonstrated to cause an increase in the number of acetylcholine receptors on the surface of cultured myotubes (New and Mudge 1986). It is possible that CGRP exerts some influence on motor mechanisms.

1.7 NEUROPEPTIDES IN ENDOCRINE GLANDS

The regulation of endocrine cells has traditionally been hypothesized to be via a humoral pathway. However, the endocrine system is further regulated by the innervation of endocrine glands. Most, if not all, endocrine glands receive nerves that appear to control both their blood flow and their secretory activity (Ojeda and Griffin, 1988). Although most of the original work on neuropeptides went into characterizing their distribution and effects within the central nervous system, there is now a growing body of information on their existence within the endocrine system. CGRP, cholecystokinin/gastrin (CCK), SP, and vasoactive intestinal polypeptide (VIP)-immunoreactivity have been identified within parafollicular cells, and nerves within the vasculature and thyroid follicles in

several mammalian species (Ahrén *et al.* 1980, 1983; Grunditz *et al.* 1986). VIP causes secretion of iodothyronine, while VIP, SP, and CCK cause a rapid and transient release of calcitonin (Ahrén *et al.* 1980, 1983).

CGRP and VIP-immunoreactive nerves have been identified within the pancreatic islets of all mammalian species investigated (Bishop *et al.* 1980; Larsson *et al.* 1978; Pettersson *et al.* 1986; Sternini and Brecha 1986). Buffa *et al.* (1977) identified VIP-immunoreactive cells in the islets of dog, guinea-pig, and man, which were distinct from the α-, β-, δ-, and the pancreatic polypeptide-cells. Bishop *et al.* (1980) identified CGRP-immunoreactive cells in the periphery of the islets that were identified as δ-cells in the rat, and β-cells in the mouse. CGRP has been shown to have a physiological effect on islet cells by suppressing insulin secretion (Pettersson *et al.* 1986).

Morel *et al.* (1982) found VIP-immunoreactivity within prolactin-secreting cells of the anterior hypophysis. VIP has been demonstrated to increase the secretion of luteinizing hormone and prolactin, but not adrenocorticotropin, follicle stimulating hormone or growth hormone (Rotsztejn *et al.* 1980; Vijayan and McCann 1979).

Neuropeptide Y (NPY)-immunoreactivity has been localized within cells of the renal medulla (Lundberg *et al.* 1986; Varndell *et al.* 1984) and within cortical nerves (Varndell *et al.* 1984). Only recently have there been any reports of neuropeptides in the parathyroid gland. Zabel *et al.* (1987) identified nerves

containing CGRP within the parathyroid stroma in rat, guinea pig, and man, as well as in the parenchyma of man and rat only. No CGRP nerves were observed in rabbit parathyroid glands. The presence of CGRP has not been looked for in bovine parathyroid glands. Joborn *et al.* (1991) found VIP enhanced cAMP release and caused a dose-dependent stimulation of PTH secretion from single bovine parathyroid cell suspensions. Consistent with the effects of the catecholamines, VIP was found to have a greater effect on PTH secretion at 0.5 mM Ca^{++} than 2.0 mM Ca^{++}.

1.8 OBJECTIVES

It is apparent from the previous review that peptidergic neurons have a wide distribution in the endocrine system, and can influence hormone secretion in many glands. Zabel *et al.* (1987) identified neuropeptides in the parathyroid glands of rat, guinea-pig, and man. However, no characterization of their presence in the bovine parathyroid gland has been attempted. It was thought to be likely that CGRP and SP would be present within nerves previously identified within the bovine parathyroid gland (Capen *et al.* 1965; Jacobowitz and Brown 1980). The first objective of this research project was therefore to investigate the presence of CGRP and/or SP in the bovine parathyroid gland. This problem was approached using indirect immunohistochemistry. CGRP and SP were visualized under a fluorescence microscope by fluorophore-conjugated

secondary antibodies directed against CGRP and SP specific antibodies. The possibility that the two neuropeptides were co-localized to nerve fibres within the gland was also investigated by double-staining parathyroid gland sections sequentially for the two neuropeptides.

The second objective of this study was to examine the effects of CGRP and SP on the modulation of PTH secretion. Primary bovine parathyroid cell cultures were employed for this investigation. Cultures were incubated with CGRP and SP to investigate their effects on PTH secretion at varying Ca^{++} concentrations. PTH secretion from the cultures was quantified using a radioimmunoassay.

In summary, the intent of this project was to investigate the immunohistochemical localization of CGRP and SP in the parathyroid gland, and to investigate the effects of these neuropeptides on PTH secretion. By completing this project, I hope the distribution and effects of CGRP and SP on the parathyroid gland will help broaden our knowledge of the roles of neuropeptides in the endocrine system. The more we know about the distribution and effects of neuropeptides, the closer we can come to understanding the global picture of their role in the physiological modulation of endocrine function.

MATERIALS and METHODS

2.1 IMMUNOHISTOCHEMISTRY

2.1.1 Antisera

Three primary (unconjugated) antisera were used in this study: (**1**) Polak's rabbit polyclonal antiserum 1209 raised against rat CGRP (rCGRP) (Gibson *et al.* 1984), (**2**) Copper's rabbit polyclonal antiserum R1 raised against human CGRP (hCGRP) (Carlton *et al.* 1987), and (**3**) Pel-Freez Biologicals' (Rogers, AR, USA) rat monoclonal antibody NC1/34 HL raised against SP (Cuello *et al.* 1979). The three fluorophore-conjugated secondary antisera were: (**1**) goat anti-rabbit IgG (fluorescein isothiocyanate (FITC)-conjugated), (**2**) goat anti-rabbit IgG (tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated), and (**3**) goat anti-rat IgG (FITC-conjugated) purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antisera were diluted to their final working concentration of 1:800 for all primary antisera, and 1:50 for FITC-labelled IgGs, and 1:100 for TRITC-labelled IgG secondary antisera with 0.05 M phosphate-buffered saline (PBS, pH 7.4) containing 0.3% Triton X-100 (PBS-TX).

2.1.2 Tissue Preparation

Twenty superior parathyroid glands were collected at a local abattoir from steers and heifers (*Bos bostaurus*) up to two years of age, within 15 min after death. Glands were immediately trimmed of all excess fat and connective tissue, cut in half longitudinally, and immersed in Zamboni's fixative (Zamboni and De

Martino 1967) on ice for 30-60 min, and stored for 24 h at 4°C. The glands were then washed 3 x 15 min in PBS on a shaker, left 24 h at 4°C in a solution of 30% sucrose and 0.1% sodium azide in 0.1 M phosphate buffer (PB), embedded in 10% sucrose and 5% agarose in 0.1 M PB, frozen in liquid nitrogen, and cut into 15 μm serial sections using a cryostat at -24°C. Sections were thaw-mounted onto gelatin-coated slides (1% gelatin and 0.1% chromium potassium sulphate) and stored at -20°C.

2.1.3 Immunohistochemical Procedure

The indirect immunofluorescence method of Coons *et al.* (1955) was used to localize the neuropeptides. Slides were washed 3 x 10 min in PBS on a shaker, aspirated dry, and 100 μl of blocking serum (1% normal goat serum) was added to each slide for 30 min, washed off, and 100 μl of the final concentration of primary antiserum was added. Slides were incubated for 24 h at room temperature, washed 3 x 10 min in PBS on a shaker, and then aspirated dry. Slides were then incubated for 2 h with 100 μl of an FITC-labelled secondary antiserum. Slides were then washed 3 x 15 min in PBS on a shaker, mounted in 4:1 glycerin to water and 0.4% n-propyl gallate, and cover-slipped. Slides were viewed and photographed on Ilford HP5 film under a fluorescence microscope.

Double-staining was carried out by sequentially staining for the two neuropeptides. Slides were incubated with the anti-rCGRP antiserum, 1209,

followed by TRITC-labelled secondary antiserum, as mentioned above. The slides were subsequently incubated with the anti-SP antiserum, NC1/34 HL, followed by FITC-labelled secondary antiserum.

2.1.4 Specificity of Antisera

For preabsorption controls antisera were diluted to 1:800 and preabsorbed for 24 h at 4°C with synthetic rCGRP, hCGRP, or SP (Sigma Chemical Co.), at various concentrations. The preabsorbed antisera were used in place of the regular antisera in the immunohistochemistry procedure above. Replacing either the primary or secondary antisera with PBS-TX in the incubation step gave negative results for all antisera.

In the co-localization procedure, no specific staining was observed when antiserum NC1/34 HL was incubated with the TRITC-labelled antiserum. However, the FITC-labelled antiserum cross-reacted with antiserum 1209. By incubating slides with antiserum 1209, followed with TRITC-labelled antiserum, the FITC-labelled antiserum was unable to bind antiserum 1209, and therefore, slides were stained for CGRP prior to staining for SP (Mortimer *et al.* 1990). Neither the FITC fluorescence nor the TRITC fluorescence bled through into the other fluorophore's filter range.

2.2 Cell Culture

2.2.1 Culture Media

(1) Sterile saline was made using 9 g/l NaCl at pH 7.40. (2) Wash medium was made using Hanks' Balanced Salts Solution (Sigma Chemical Co.) containing 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 4.2 mM $NaHCO_3$, 2.0 mM Ca^{++}, 0.8 mM Mg^{++}, 10 mg/ml gentamicin sulfate, 10 000 U/ml Penicillin G, 10 mg/ml Streptomycin Sulphate, and 10 mg/ml amphotericin B, pH 7.40. (3) Digestion medium was made using Ca^{++} and Mg^{++} free Waymouth MB 752/1 Medium (Gibco BRL, Burlington, Ontario, Canada) supplemented with 15 mM HEPES, 4.4 mM $NaHCO_3$, 2.0 mM Ca^{++}, 0.75 mM Mg^{++}, 10 mg/ml gentamicin sulfate, 10 000 U/ml penicillin G, 10 mg/ml Streptomycin, and 10 mg/ml amphotericin B, pH 7.40. (4) Culture medium was made using Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL) containing 15 mM HEPES, 44 mM $NaHCO_3$, 1.25 mM Ca^{++}, 0.75 mM Mg^{++}, 0.87 μM bovine insulin (Sigma Chemical Co.), 64 nM bovine transferrin (Sigma Chemical Co.), 10 mg/ml gentamicin sulfate, 10 mg/ml amphotericin B, pH 7.40.

2.2.2 Collagenase Purification

Collagenase for gland dispersion was purified according to the method of Schultz *et al.* (1980). 1.00 g of Clostridium collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA) was dissolved in 10 ml column buffer (10 mM HEPES, 2.0 mM $CaCl_2$, pH 7.40), and applied to a 5 cm x 60 cm column

(Pharmacia Fine Chemicals, Piscataway, NJ, USA) containing Sephadex G-100 superfine gel (Pharmacia Fine Chemicals) at 4°C, and eluted at a flow rate of 17 ml/h. Fractions were collected at 30 min intervals and ultraviolet absorbance read at 280 nm to determine protein content. The first protein peak after the void volume, representing purified collagenase, was pooled, lyophilized, brought up in digestion medium at a concentration of 12 000 U/ml, and stored at -70°C before use.

2.2.3 Gland Collection and Digestion

Gland collection and digestion was modified from MacGregor *et al.* (1983). Thirty five superior parathyroid glands were collected at a local abattoir from steers and heifers (*Bos bostaurus*), up to two years of age, within 15 min after death. Glands were placed in 75 ml sterile wash medium on ice and transported to the laboratory. Glands were washed for 2 min in a solution of 50:50 sterile 0.15 mM NaCl to 70% ethanol, followed by three washes in sterile saline, then two washes in wash medium while on ice. Glands were trimmed of all excess fat and connective tissue, then sliced into 10 μm sections with a Stadie-Riggs tissue slicer (Thomas Scientific Inc., Swedesboro, NJ, USA). The tissue was placed in a 100 ml beaker containing wash medium and further minced using fine surgical scissors and the fat allowed to float to the surface before being decanted off. This was carried out several times until no fat was visible. The tissue was transferred to a 500 ml erlenmeyer flask with 60 ml digestion medium

containing 400 U/ml purified collagenase, 150 μg/ml papain (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA), and 40 μg/ml deoxyribonuclease II (Sigma Chemical Co.). The tissue was then digested for 6 h in a 37°C Dubnoff metabolic shaking incubator (Precision Scientific, Inc., Chicago, IL, USA) at 140 rpm. The tissue was vigorously pipetted every 30 min with a 10 ml serological pipette fitted to a 10 ml syringe to further aid in dispersing the tissue. After all the cells were dispersed, they were placed in 50 ml conical tubes and centrifuged at 1000 rpm for 10 min. The medium was aspirated off, the cells resuspended in wash medium, and centrifuged at 800 rpm for 5 min. This was repeated 4 times, the last two times using culture medium. A 100 μl aliquot was removed and 10 μl of 0.5% Trypan Blue added. Cell counts were determined using a haemocytometer and viability assessment made by Trypan Blue exclusion. The cells were seeded at a density of 1.0×10^6 viable cells/ml/well in 24 well plates (Nalgene Co., Rochester, NY, USA) using culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL) and placed in a humid 37°C incubator (Model #3331, National Appliance Co., Portland, OR, USA) in an atmosphere of 5% CO_2 and 95% air.

2.3 INCUBATIONS

2.3.1 Chemicals and Media

Synthetic α-hCGRP (80.4% peptide) and SP (73.0% peptide) were purchased from Bachem Inc. (Torrance, CA, USA), and both peptides were greater than 99% pure. The lyophilized powders were diluted to 10^{-3} M using sterile distilled water, with the concentrations calculated according to the peptide content, aliquoted into Ependorff micro-centrifuge tubes, and stored at -70°C before use. The purity of the peptides was verified using high performance liquid chromatography with detection by absorbance at 210 nm. Culture medium was made using DMEM (Gibco BRL) containing 15 mM HEPES, 44 mM $NaHCO_3$, either 0.5, 1.25, or 2.0 mM Ca^{++}, 0.75 mM Mg^{++}, 10 mg/ml gentamicin sulfate, and 10 mg/ml amphotericin B, pH 7.40.

2.3.2 Procedure

Between 2-3 d after seeding, culture medium was aspirated from each well, and replaced with 1200 μl of the equilibrated incubation medium. 200 μl of the medium was immediately removed (time 0 min). 200 μl of medium was removed at either 30 min or 45 min, and replaced with 200 μl of equilibrated medium. At either 60 min or 90 min, 800 μl of the medium was removed. All the medium was placed into Ependorff microcentrifuge tubes and spun for 1 min in a micro-centrifuge. Medium was then removed from the tubes and placed in 75 mm x 150 mm test tubes containing 10% 0.5 M acetic acid (v/v) and stored at -20°C.

2.4 RADIOIMMUNOASSAY

An equilibrium radioimmunoassay (RIA) of secreted PTH in the culture medium was performed using a guinea-pig antiserum (GP-467) raised against a crude preparation of bovine PTH (bPTH) (TCA powder, Inolex Laboratories, Grenwood, Il, USA), which has been characterized as having a detection preference for intact PTH (Hanley *et al.* 1985). A final antibody dilution of 1 to 140 000 in the assay was used in 0.01 M Veronal buffer, 0.01 M EDTA, pH 8.6, containing PTH free human plasma (1:5 v/v) as the assay buffer. Standards and samples were assayed in triplicate, using a Gilson diluter. 25 μl intact bPTH (Bachem Inc.) was used for the reference standard. 50 μl radiolabelled bPTH (100 cpm/μl) was added to each tube and incubated for 4 days at 4°C. Bovine PTH was iodinated with ^{125}I (Amersham Canada Ltd., Oakville, Ontario, Canada) by chloramine T according to the method of Roos and Deftos (1979). Bound and free tracer were separated by the double antibody method. 100 μl of guinea-pig serum (1:300) and 100 μl of goat anti-guinea pig antiserum (1:16) in 0.01 M veronal buffer were added to each tube and incubated at 4°C for 24 h. Tubes were spun at 3000 rpm for 30 min at 4°C in a DCP-6000 centrifuge (IEC, Needham Heights, MA, USA), aspirated, and counted on an LKB-Wallac 1274 RiaGamma counter (Cambridge, England) using the spline-function method (Rawlins and Yrjönen, 1978).

2.5 STATISTICS

Each experimental condition was repeated on 4 wells within an experiment and experiments repeated at least 3 times. Data are expressed as mean ± standard deviation (SD) of 4 replicates. After conversion to amount of PTH secreted above time 0 min, secretion data were pooled and analyzed by the non-parametric two-tailed Mann-Whitney *U*-test for small samples (Siegel and Castellan 1988). The criterion for significance was set at $P < 0.05$ for all comparisons.

RESULTS

3.1 IMMUNOHISTOCHEMISTRY

3.1.1 Calcitonin Gene-Related Peptide

Rat CGRP-like immunoreactive (CGRP-LI) nerve fibres with varicosities were observed within the parathyroid gland using Polak's anti-rCGRP antiserum. Numerous rCGRP-LI fibres were observed close to or within the walls of blood vessels. Most arteries and arterioles observed contained rCGRP-LI fibres within the tunica adventitia and often closely apposed to the tunica media (Figure 3.1A). The fibres showed a large variation in size. No rCGRP-LI fibres were associated with any veins or capillaries. Rat CGRP-LI fibres were not restricted to the arterial vasculature, but appeared also throughout the stroma of the gland. Most of the fibres were located in large stromal areas containing arteries. Both straight and tortuous, as well as large and small fibres were found here (Figure 3.1A).

Frequently slender rCGRP-LI fibres were found winding through small stromal areas between individual parenchymal lobules, apparently not associated with any vessels. Many of these fibres were closely apposed to the parenchyma. Some parenchymal lobules were devoid of encompassing fibres, while others appeared to be almost completely surrounded by fibres (Figure 3.1B). They had a characteristically patchy distribution with areas totally devoid of immunoreactive fibres. However, none of the fibres were found to enter the parenchyma and

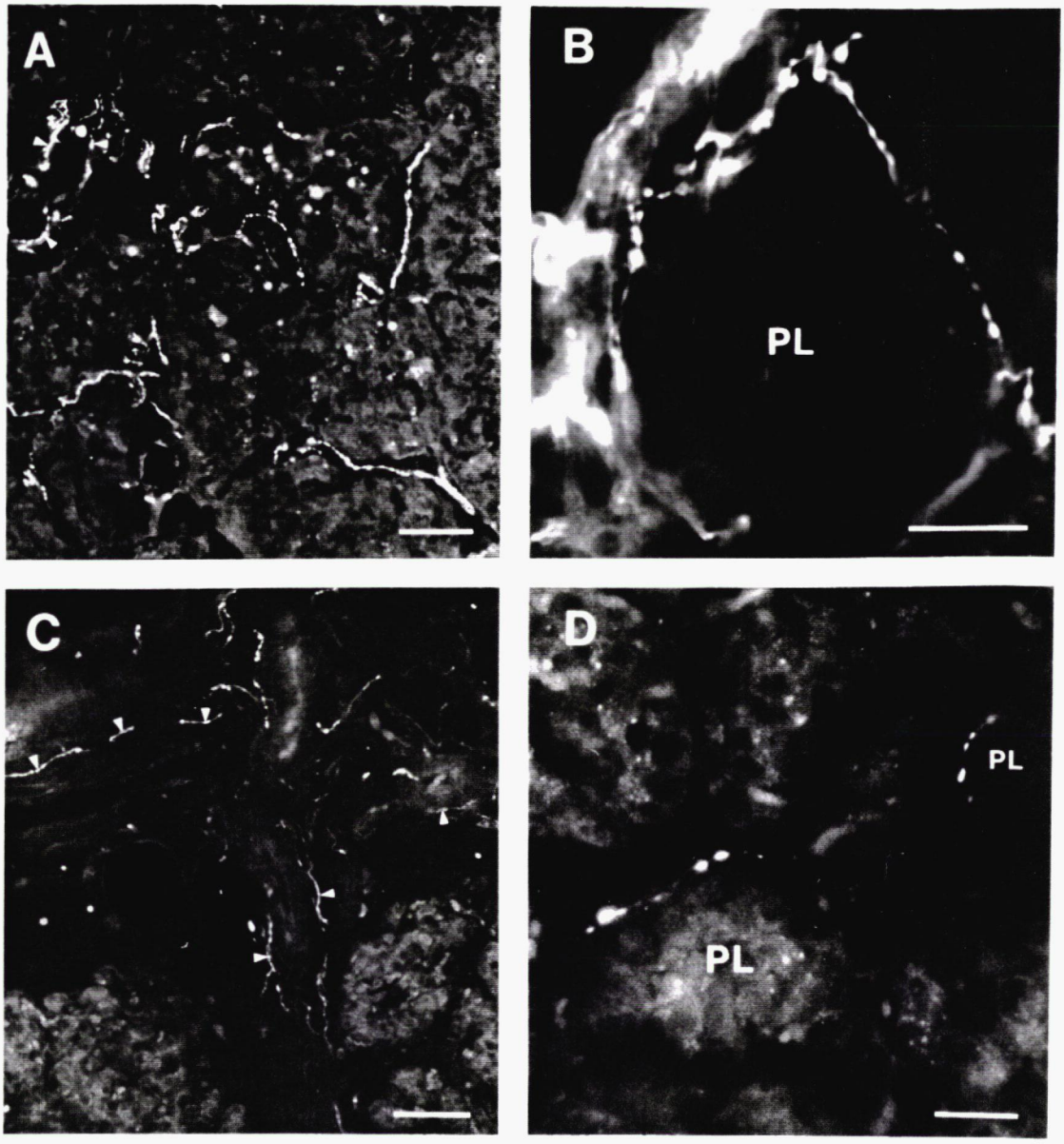


Figure 3.1 Immunofluorescence localization of CGRP-LI in the bovine parathyroid gland using Polak's anti-rCGRP antiserum 1209 (**A**, **B**) and Cooper's anti-hCGRP antiserum R1 (**C**, **D**). **A**, **C** Immunoreactive nerve fibres are located within the walls of small muscular arteries (*arrowheads*), and within the stroma of the gland independent of the vasculature. **B**, **D** Immunofluorescence localization of CGRP-LI nerve fibres encircling parenchymal lobules (*PL*). No immunoreactive fibres are seen penetrating into the parenchyma. **A**, **C** x 62.5, **B** x 250, **D** x 125. *Bars*: 50 μm (**A**, **B**), 20 μm (**C**, **D**).

synapse with the chief cells.

Similar results were obtained using Cooper's antiserum to hCGRP. The pattern and distribution of hCGRP-LI nerve fibres were the same as those observed using antiserum to rCGRP. That is, hCGRP-LI fibres were observed within arteries and large stromal areas (Figure 3.1C) and encircling the parenchymal lobules (Figure 3.1D), but no contacts were observed between fibres and chief cells.

Preabsorption controls were carried out according to the methods described. Discernable staining diminished from 100% to 0% within a 100-fold increase in concentration of antigen for both Polak's and Cooper's antisera (Table 3.1). Both antisera showed the same levels of staining when preabsorbed with 10^{-5} M SP as with unpreabsorbed antisera, indicating no cross-reactivity of either CGRP antisera with SP.

3.1.2 Substance P

Substance P-like immunoreactive (SP-LI) fibres were also identified in bovine parathyroid glands. The SP-LI fibres appeared to be similar to CGRP-LI fibres in diameter and the presence of varicosities, and had a staining intensity and distribution comparable to those for CGRP. The immunoreactive fibres were observed within the stroma of the gland (Figure 3.2A). Many of the fibres were found within the tunica adventitia closely apposing the tunica media in arteries and arterioles (Figure 3.2A). Similarly to the CGRP-LI fibres, SP-LI fibres were

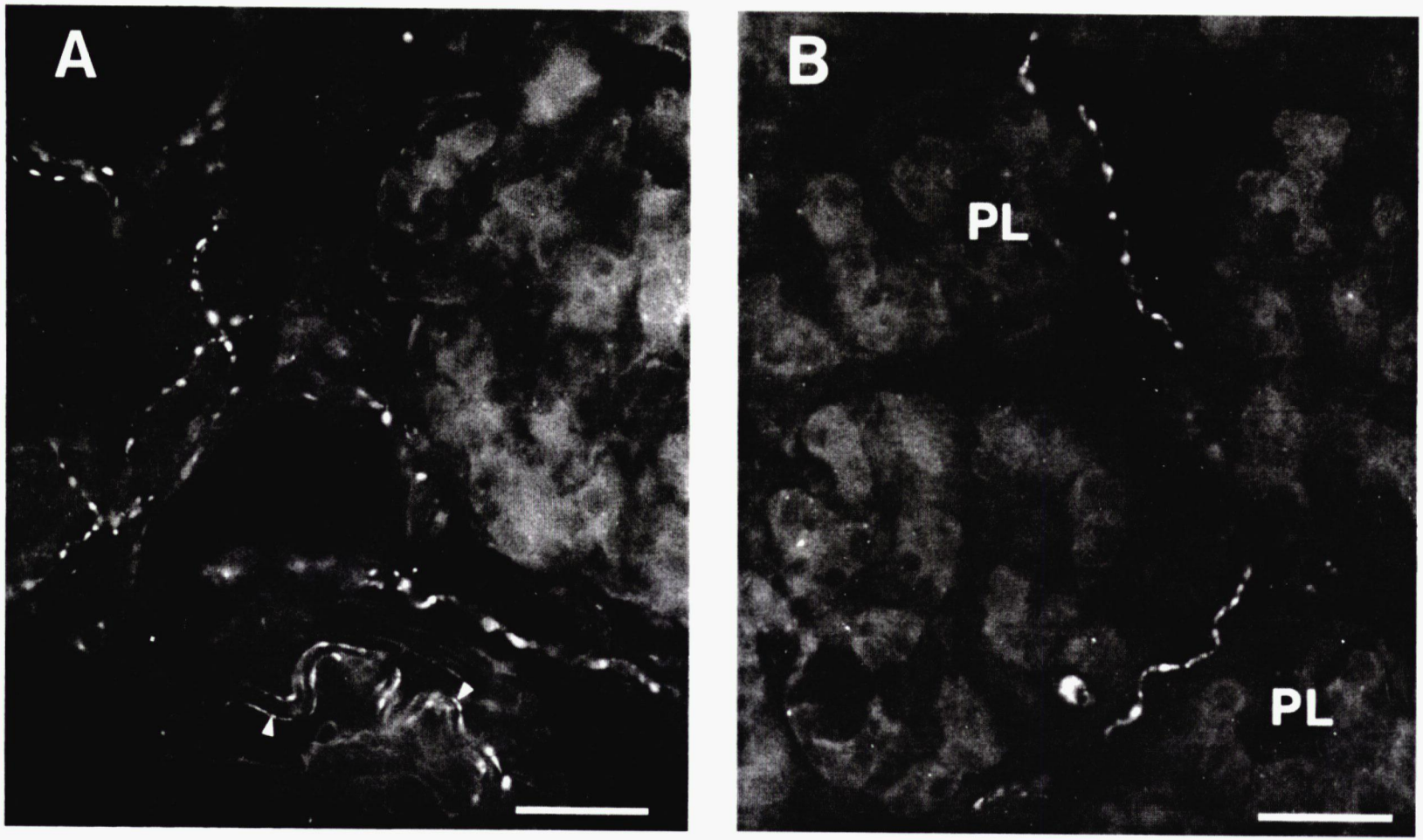
Table 3.1 Preabsorption controls for the immunohistochemical antibodies.

Antisera[a]	Antigen	Concentration of antigen[b]						
		10^{-11}M	10^{-10}M	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
rCGRP	rCGRP	++	+	–	–	–	–	–
	hCGRP				++	–	–	–
	SP							++
hCGRP	rCGRP				++	++	++	–
	hCGRP		++	++	+	–	–	–
	SP							++
SP	rCGRP							++
	hCGRP							++
	SP	++	++	++	+	–	–	–

[a] Antisera used were Polak's anti-rCGRP antiserum 1209, Cooper's anti-hCGRP antiserum R1, and Pel-Freez Biologicals' anti-SP antiserum NC1/34 HL preabsorbed with synthetic rCGRP, hCGRP, and SP.

[b] The binding is expressed as: ++, 100% maximum; +, 50% maximum; –, 0% maximum.

Figure 3.2 Immunofluorescence localization of SP-LI in the bovine parathyroid gland using Pel-Freez Biologicals' anti-SP antibody NC1/34 HL. **A** Immunoreactive nerve fibres are located within the arterial vasculature (*arrowheads*), and within the stroma of the gland where they cannot be followed along any vessels. **B** Immunoreactive fibres surrounding the parenchymal lobules (*PL*). No immunoreactive fibres are seen penetrating into the parenchyma. x 125. *Bars*: 30 μm.


A
B
PL
PL

also found in close proximity to the parenchymal lobules (Figure 3.2B). However, the fibres did not enter the parenchyma nor made contact with any chief cells. Antiserum NC1/34 HL preabsorbed with synthetic SP gave results similar to those for Cooper's antiserum preabsorbed with hCGRP (Table 3.1). Staining intensity decreased from maximal to zero within a 100-fold increase in the concentration of antigen. Antiserum NC1/34 HL preabsorbed with 10^{-5} M rCGRP or hCGRP showed the same levels of specific staining as with unpreabsorbed antiserum.

3.1.3 Co-localization of Calcitonin Gene-Related Peptide and Substance P

Since both CGRP-LI and SP-LI fibres were found to have approximately the same distribution in bovine parathyroid glands, I attempted to determine whether the two neuropeptides were present in the same fibres. Sections were stained with antisera 1209 and NC1/34 HL. Fibres containing both rCGRP-LI and SP-LI were observed in double-staining experiments. All of the fibres showed identical staining for both CGRP and SP (Figure 3.3).

3.2 EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P ON PARATHYROID HORMONE SECRETION

3.2.1 Calcitonin Gene-Related Peptide

The effects of hCGRP on PTH secretion from bovine parathyroid cell cultures was investigated. CGRP, at concentrations between 10^{-8} M and 10^{-5} M, had no

Figure 3.3 Immunofluorescence co-localization of CGRP-LI and SP-LI in bovine parathyroid gland using Polak's anti-rCGRP antiserum 1209 and Pel-Freez Biologicals' anti-SP antibody NC1/34 HL. CGRP-LI nerve fibres seen under a TRITC filter (**a**) and SP-IR nerve fibres seen under a FITC filter (**b**). All immunoreactive fibres within the gland are seen under both filters indicating that all fibres contain both neuropeptides. x 125.

b
a

significant effect on PTH secretion at 45 min after its addition into cultures in the presence of a normal physiological calcium concentration of 1.25 mM Ca^{++} (Figure 3.4). Similar results were also seen after the cultures had been incubated for 90 min.

Cultures incubated in low calcium concentrations (0.5 mM Ca^{++}) also showed similar results. Cultures incubated at CGRP concentrations between 10^{-8} M and 10^{-5} M for 60 min showed no significant change in their rate of PTH secretion over control cultures not exposed to CGRP (Figure 3.5). In the presence of 2.0 mM Ca^{++}, cultures also showed no change in PTH secretion when incubated with CGRP at concentrations of 10^{-8} M to 10^{-5} M (Figure 3.6).

3.2.2 Substance P

The bovine parathyroid cultures also did not respond to the presence of SP in the incubation medium. Figure 3.7 shows a dose response curve for cultures incubated at 1.25 mM Ca^{++} in the presence of SP at concentrations between 10^{-8} M and 10^{-5} M. At 45 min and 90 min, the cultures did not change their rate of PTH secretion from that of cultures incubated at 1.25 mM Ca^{++} in the absence of SP.

SP, at concentrations between 10^{-8} M and 10^{-5} M, also had no significant effect on PTH secretion at 0.5 mM Ca^{++} for up to 60 min (Figure 3.8). Similar results were also found in cultures incubated with SP (10^{-8} M to 10^{-5} M) at 2.0 mM Ca^{++} (Figure 3.9) for up to 60 min.

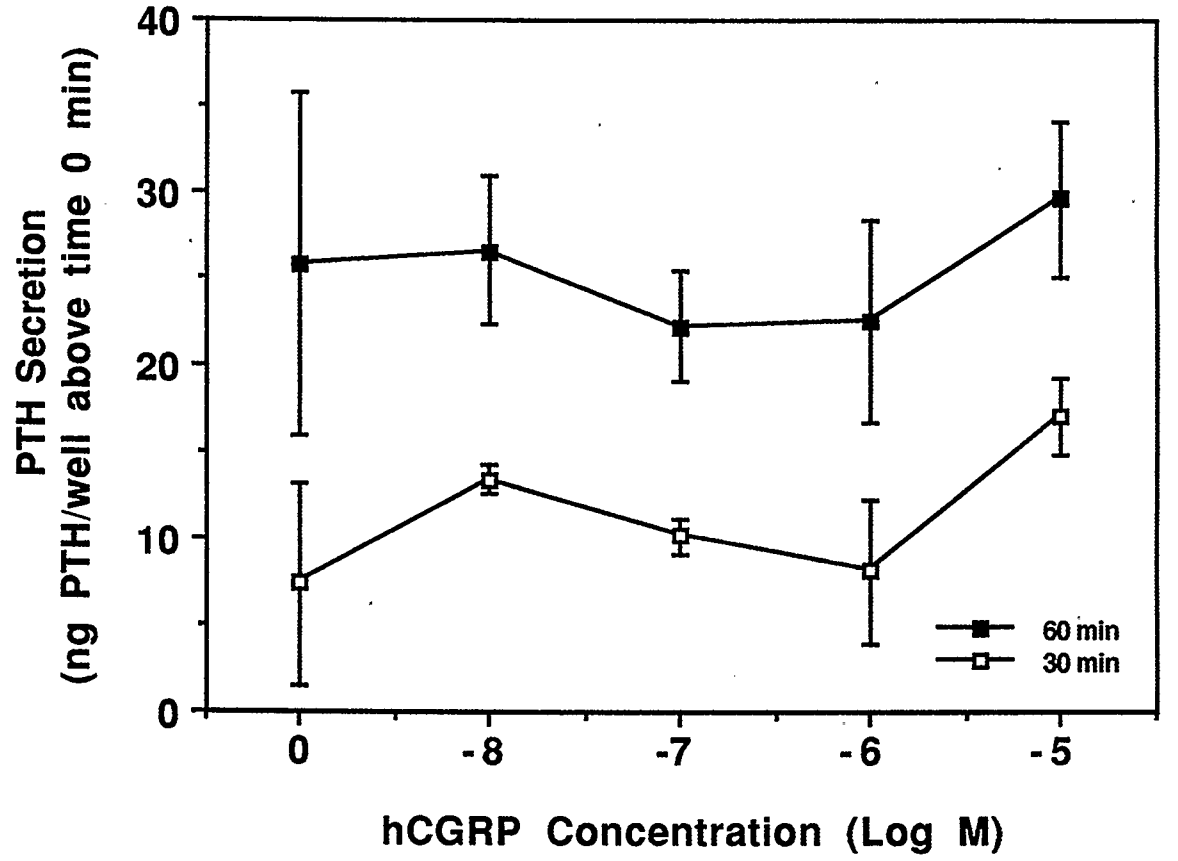


Figure 3.4 Dose response curve for hCGRP effect on PTH secretion from bovine parathyroid cell cultures in the presence of 1.25 mM Ca^{++}.

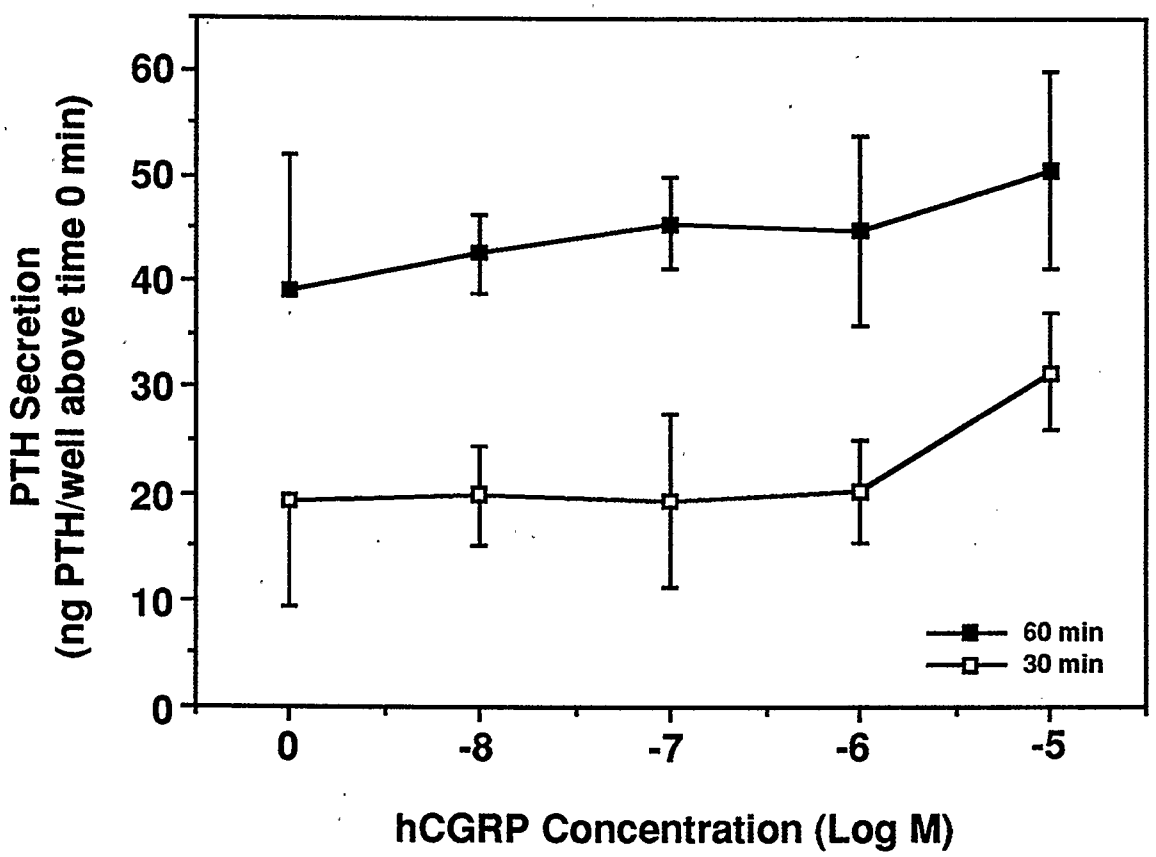


Figure 3.5 Dose response curve for hCGRP effect on PTH secretion from bovine parathyroid cell cultures in the presence of 0.5 mM Ca^{++}.

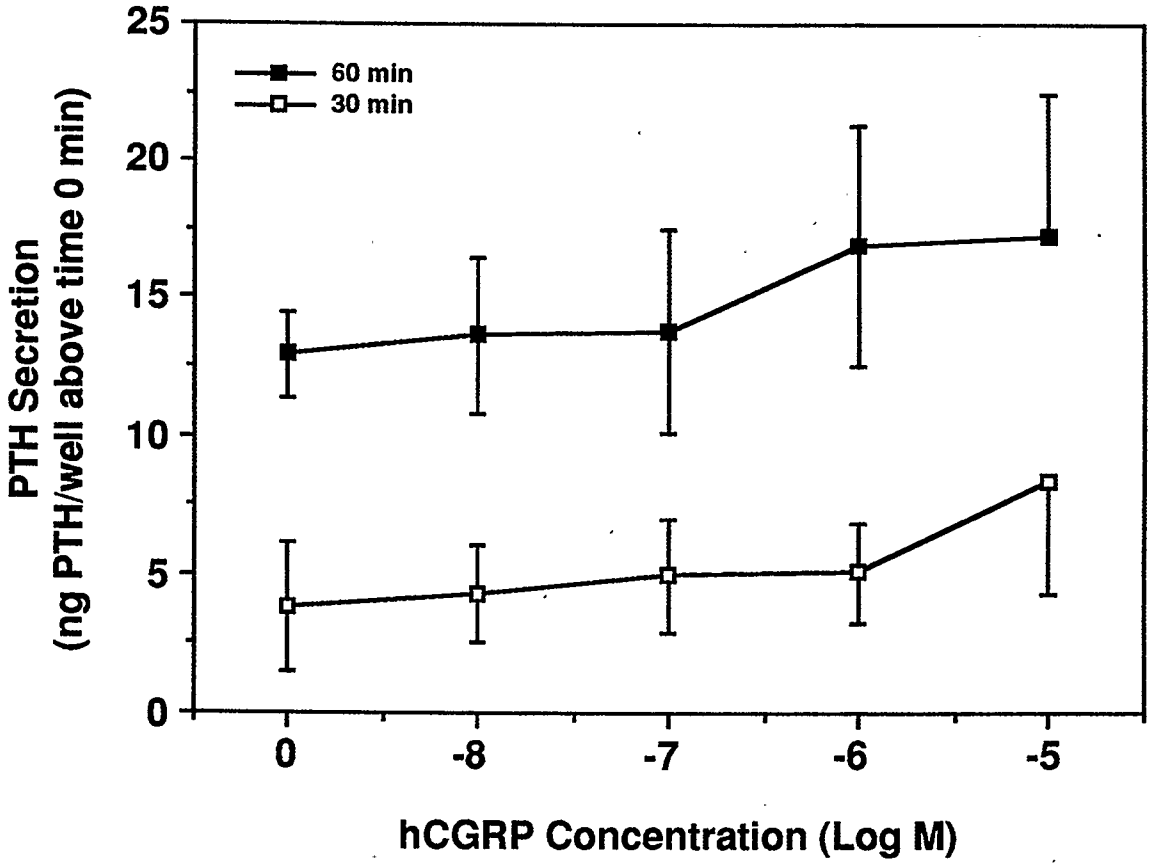


Figure 3.6 Dose response curve for hCGRP effect on PTH secretion from bovine parathyroid cell cultures in the presence of 2.0 mM Ca^{++}.

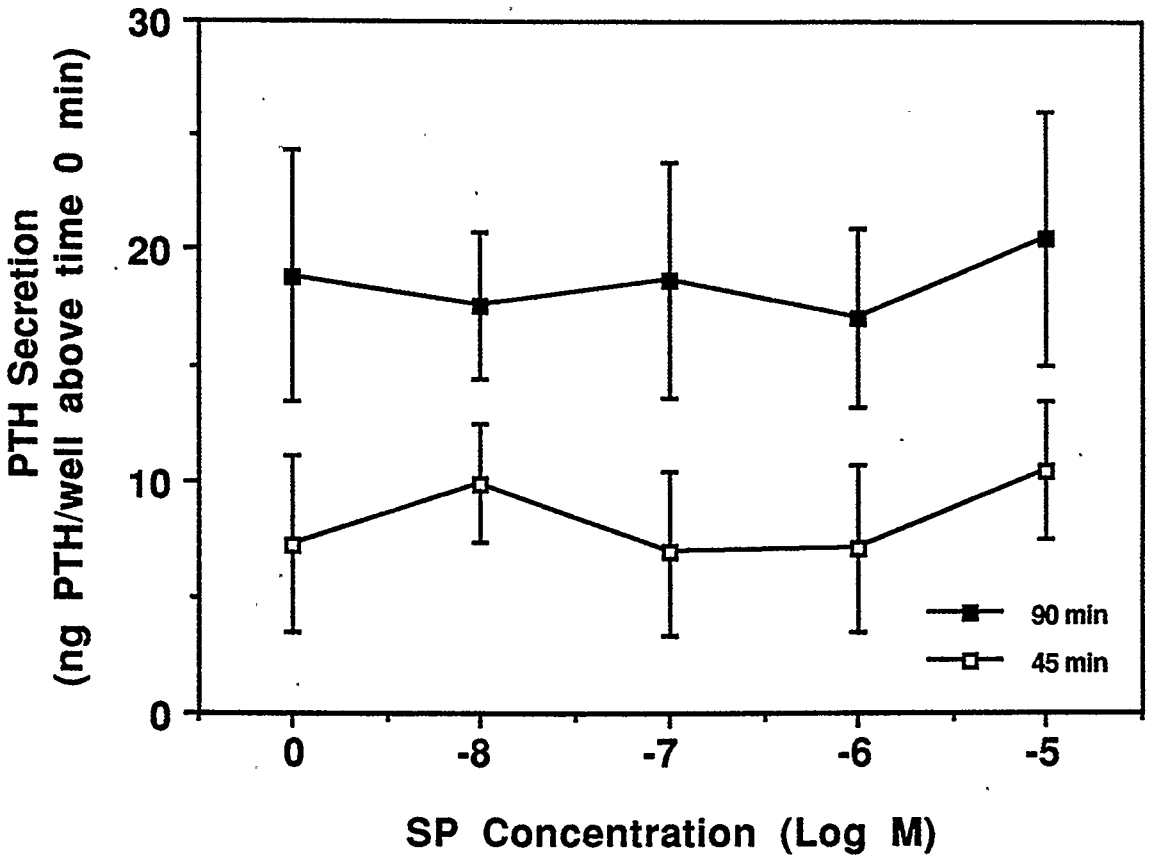


Figure 3.7 Dose response curve for SP effect on PTH secretion from bovine parathyroid cell cultures in the presence of 1.25 mM Ca^{++}.

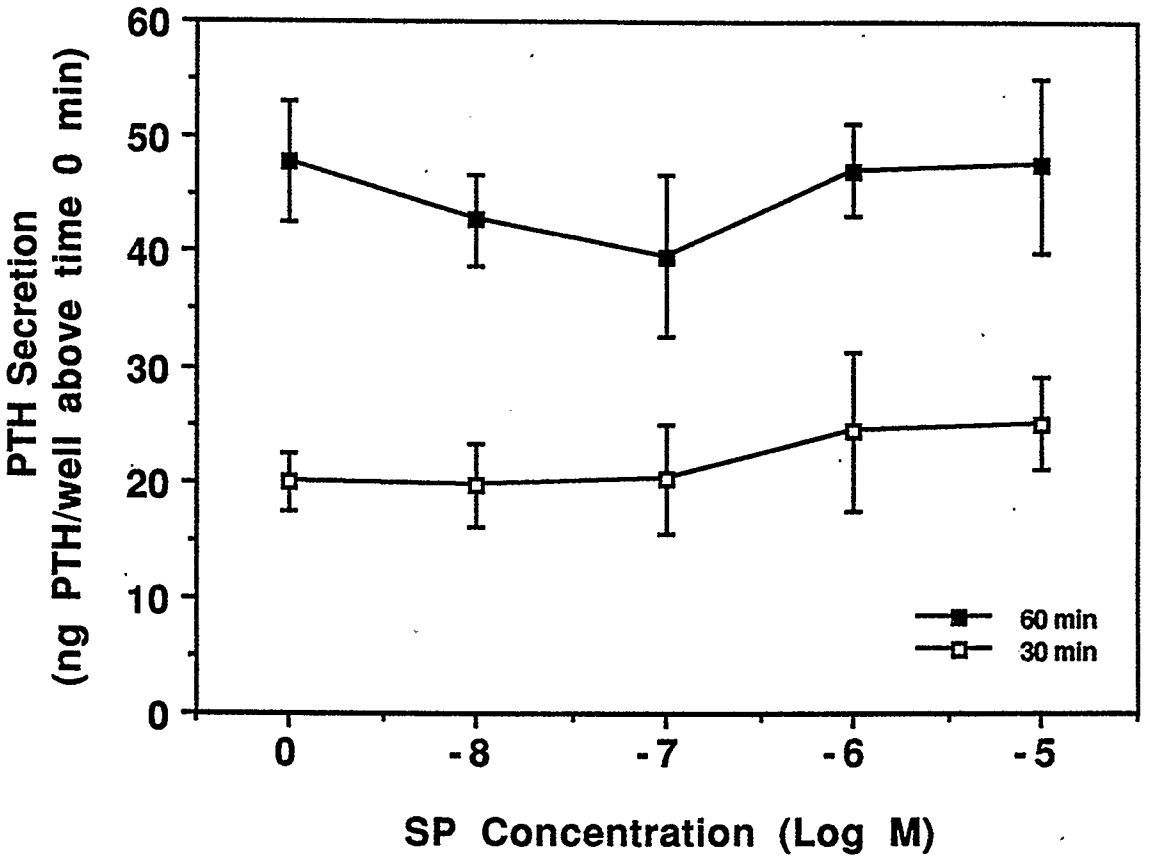


Figure 3.8 Dose response curve for SP effect on PTH secretion from bovine parathyroid cell cultures in the presence of 0.5 mM Ca^{++}.

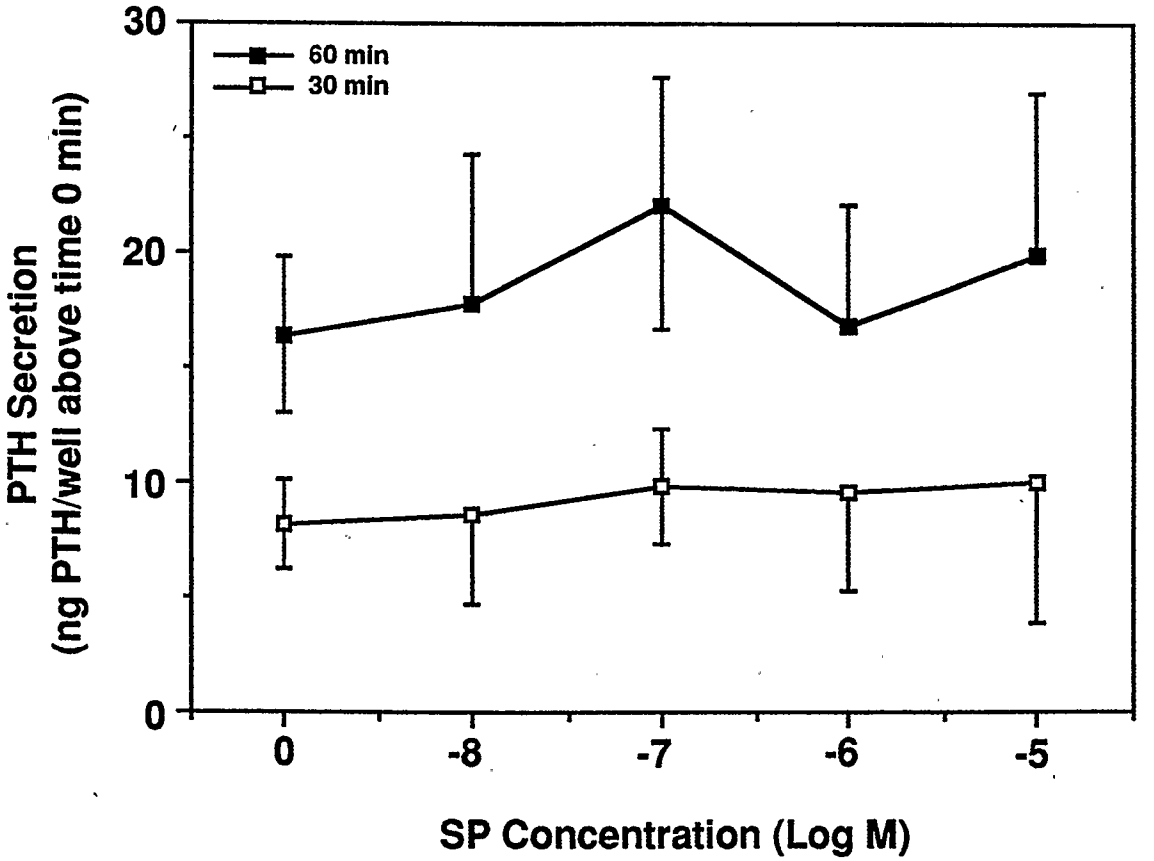


Figure 3.9 Dose response curve for SP effect on PTH secretion from bovine parathyroid cell cultures in the presence of 2.0 mM Ca^{++}.

3.2.3 Calcitonin Gene-Related Peptide and Substance P

The possibility that CGRP and SP are required together to have an effect on PTH secretion was also investigated. The results were the same as when CGRP and SP were incubated separately. Concentrations of CGRP and SP between 10^{-10} M to 10^{-6}M had no significant effect on PTH secretion at 1.25 mM Ca^{++} at either 30 min or 60 min (Figure 3.10).

3.2.4 Responsiveness to Known Secretagogues

The cultures were responsive to calcium. Cultures exposed to 0.5 mM Ca^{++} showed a significant increase in PTH secretion from cultures exposed to 2.0 mM Ca^{++} (Table 3.2). At 1.25 mM Ca^{++}, PTH secretion from cultures incubated with isoproterenol (10^{-6} M) or dopamine (10^{-5} M) were significantly higher than control cultures incubated without the catecholamines (Table 3.2).

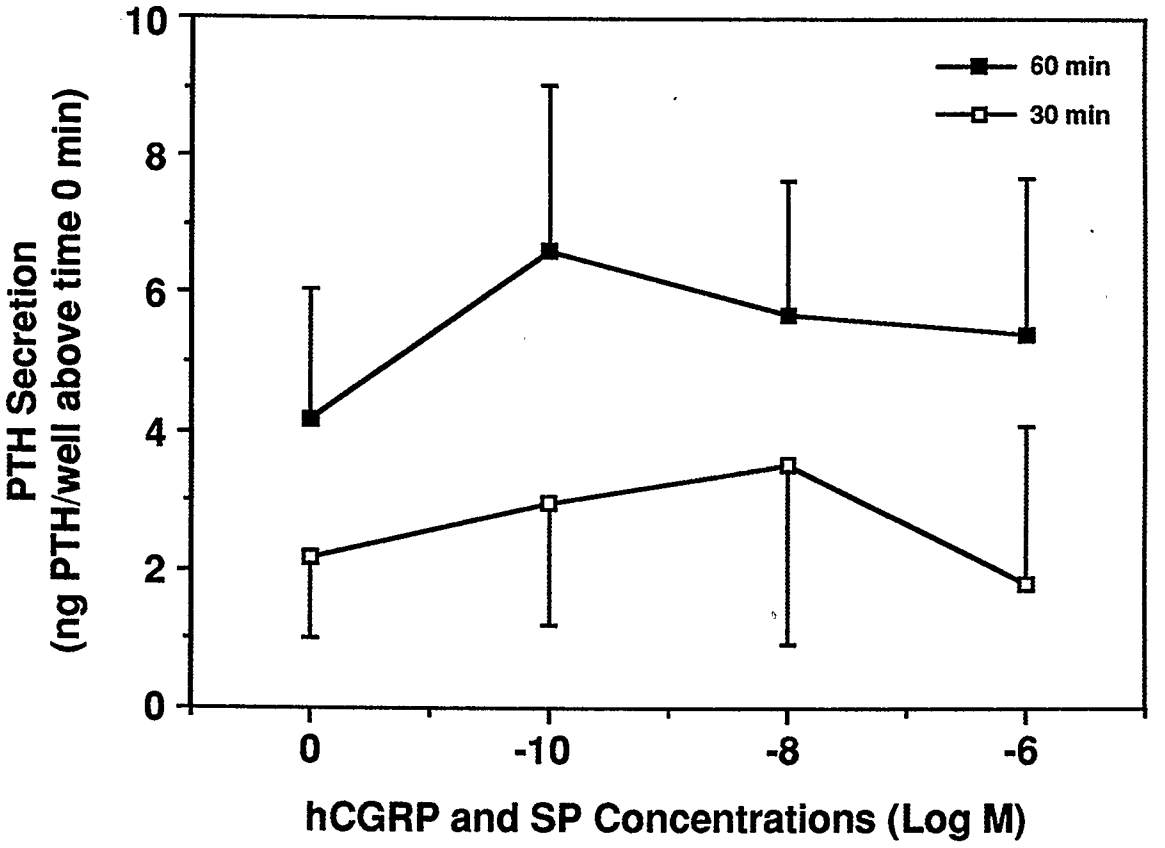


Figure 3.10 Dose response curve for the effects of hCGRP and SP on PTH secretion from bovine parathyroid cell cultures in the presence of 1.25 mM Ca^{++}.

Table 3.2 Responsiveness of the parathyroid cell cultures to calcium and the catecholamines isoproterenol, and dopamine.

	Secretion of PTH (ng PTH/well above time 0 min)	
	45 min	90 min
0.5 mM Ca^{++} (8)	20.04 ± 7.31	40.32 ± 8.23
1.25 mM Ca^{++} (12)	16.41 ± 11.14	33.14 ± 11.10
1.25 mM Ca^{++} + 10^{-6} M Isoproterenol (12)	41.37 ± 13.28[a]	66.15 ± 22.00[a]
1.25 mM Ca^{++} + 10^{-5} M Dopamine (12)	34.09 ± 9.65[a]	64.93 ± 9.41[a]
2.0 mM Ca^{++} (8)	12.74 ± 5.95[b]	25.21 ± 11.92[b]

Data are expressed as mean ± SD. The number of cultures used are indicated in parentheses. After conversion to amount of PTH secreted above time 0 min, secretion data were pooled and analyzed by the non-parametric two-tailed Mann-Whitney *U*-test for small samples (for n=8) or large samples (for n=12).

[a] Significantly different from 1.25 mM Ca^{++}, $p < 0.05$.

[b] Significantly different from 0.5 mM Ca^{++}, $p < 0.05$.

DISCUSSION

4.1 CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P IN THE VASCULATURE

In the present study I used indirect immunohistochemistry to identify, for the first time, the presence of immunoreactive CGRP and SP within the bovine parathyroid gland. CGRP immunoreactivity was positively identified in nerve fibres within the substance of the gland. I found CGRP-LI fibres to be located within the small arteries and arterioles throughout the gland. These fibres within the arterial vasculature of the parathyroid gland formed a meshwork within the tunica adventitia. Many of the fibres were found at the tunicae adventitial-medial junction, but did not penetrate into the tunica media.

Shortly after CGRP's discovery, thin beaded CGRP-LI fibres were identified within the smooth muscle of blood vessels of the gastrointestinal tract, heart, lung, and tongue (Rosenfeld *et al.* 1983). High concentrations of CGRP-LI have been reported in the abdominal aorta, as well as the carotid, cerebral, femoral, renal, and superior mesenteric arteries (Hanko *et al.* 1985; Mulderry *et al.* 1985; Uddman *et al.* 1986; Wanaka *et al.* 1987). CGRP also has a wide distribution within small arteries and arterioles (Wanaka *et al.* 1986), which is consistent with its presence in the bovine parathyroid gland vasculature. Hanko *et al.* (1985), Uddman *et al.* (1986), and Wanaka *et al.* (1987) found CGRP-LI nerves present as a meshwork in the tunica adventitia, often making contact with, but not

penetrating the tunica media.

I did not observe any CGRP-LI fibres associated with the venous system in the bovine parathyroid gland. While high levels of CGRP have been reported in the proximal regions of the femoral and renal veins, and inferior vena cava, the nerve fibre frequency diminishes as the veins are traced peripherally (Mulderry *et al.* 1985; Uddman *et al.* 1986).

I also established for the first time the presence of SP in nerve fibres within the parathyroid gland. Like CGRP, many SP-LI fibres were found as a meshwork within the tunica adventitia in arteries and arterioles, where they frequently apposed the tunica media. In several mammalian species, almost every arterial bed investigated to date (brain, cardiovascular system, and the respiratory, gastrointestinal, urinary, and reproductive tracts) has been shown to be supplied with SP-containing fibres down to the level of the arterioles and metarterioles (Costa *et al.* 1980; Edvinsson and Uddman 1982; Furness *et al.* 1982; Reinecke *et al.* 1980; Sundler *et al.* 1977; Wharton *et al.* 1981). The SP-LI nerve networks are present in the tunica adventitia, and the tunicae adventitial-medial junction of the arterial vessels.

Like the CGRP-LI fibres, no SP-LI fibres were found associated with the venous system in the bovine parathyroid gland. SP-LI nerves have been found within large veins, but their density decreases as one traces the veins distally, becoming very sparse or absent in small veins and venules (Furness *et al.* 1982;

Reinecke *et al.* 1980; Wharton *et al.* 1981).

The distribution of SP-LI fibres within the bovine parathyroid gland was similar to that described for CGRP-LI fibres. In double-staining experiments, all fibres showed identical staining for both CGRP and SP (Figure 3.3). Many investigators have found a high level of co-localization of CGRP and SP within the same fibres in both the central and peripheral nervous systems (Gibbins *et al.* 1985; Hardebo *et al.* 1989; Lee *et al.* 1985a, 1985b; Skofitsch and Jacobowitz 1985; Wiesenfeld-Hallin *et al.* 1984). Gulbenkian *et al.* (1986) were able to demonstrate the coexistence of CGRP and SP within secretory vesicles of peripheral nerves in the guinea pig, suggesting they may be co-released from peripheral axons.

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), a pungent ingredient found in peppers of the genus *Capsicum*, is a neurotoxin (Bevan and Szolcsányi 1990; Holzer 1988; Maggi and Meli 1988). Capsaicin causes the selected depletion of neuropeptides from C fibres with no evidence of actions on non-sensory neurons and is therefore used as a marker for C fibres. It is well documented that systemic capsaicin treatment of guinea pigs and rats leads to the depletion of CGRP and SP immunoreactivity from nerve fibres in the vascular system (Barja *et al.* 1983; Duckles and Buck 1982; Duckles and Levitt 1984; Furness *et al.* 1982; Gibbins *et al.* 1985; Lundberg *et al.* 1985; Wharton *et al.* 1986). This strongly suggests a sensory origin for these nerves.

Cross-reactivity with other peptides and proteins containing amino acid sequences recognized by the CGRP and SP antisera cannot be excluded. Therefore, the expression CGRP- and SP-LI was used throughout this thesis.

It was unlikely that the anti-CGRP antibodies were cross-reacting with calcitonin. Gibson *et al.* (1984) reported no cross-reactivity between 10 nmol calcitonin/ml anti-CGRP antiserum 1209. Amylin, the major peptide component of islet amyloid commonly found in the pancreases of patients with non-insulin-dependent diabetes mellitus, shows 46% sequence homology with CGRP (Cooper *et al.* 1987). Antisera to rCGRP has also been shown to cross-react with human amylin (Clark *et al.* 1987). However, amylin and calcitonin do not appear to be present in the nervous system.

There is a possibility that the anti-SP antiserum NC1/34 HL cross-reacts with NKA or NPK, which are reported to exist in sensory nerves (Dalsgaard *et al.* 1985; Diez Guerra *et al.* 1988; Hua *et al.* 1985). RIA crossreactivity experiments indicate that NC1/34 HL recognizes a determinant located in the carboxy-terminal portion of SP (Cuello *et al.* 1979). It is possible that NC1/34 HL recognizes the carboxy-terminal tripeptide sequence common to all tachykinins, and was cross-reacting with a tachykinin other than SP in the bovine parathyroid gland.

4.2 CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P NEAR THE PARENCHYMA

I found that many of the peptidergic fibres were closely apposed to the parenchyma and not associated with any arteries. Although these fibres appeared to encircle some parenchymal lobules, I was unable to demonstrate any CGRP or SP fibres extending into the parenchyma. No synaptic contacts with the chief cells was observed either. There appears to be a species specificity to the distribution of CGRP within the parathyroid gland. The distribution of CGRP-LI fibres in man and rat are different from those in the bovine. However, the innervation of the bovine parathyroid gland with peptidergic nerves is consistent with the findings of earlier workers, Capen *et al.* (1965) and Jacobowitz and Brown (1980), who reported nerve fibres confined to the stroma.

The chief cells are likely targets for these perilobular fibres. As no intimate contact with chief cells was observed, these neuropeptides would probably have a paracrine mode of action. By diffusing into the parenchyma, CGRP and SP might interact with receptors on the chief cells to initiate a physiological response. Therefore, the short term effects of CGRP and SP on PTH secretion from primary bovine parathyroid cell cultures were investigated.

4.3 EFFECT OF CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P ON PARATHYROID HORMONE SECRETION

I was unable to demonstrate any direct effect of CGRP or SP on PTH secretion from the primary bovine parathyroid cell cultures with treatment as high as 10^{-5} M for up to 90 min at 1.25 mM Ca^{++}, and 60 min at 0.5 and 2.0 mM Ca^{++}.

SP has been co-localized with CGRP in nerve terminals. There is very little known for the significance of this co-localization. While knowledge of the actions of neuropeptides on target cells has advanced greatly in recent years, the majority of studies have focused on actions of single substances, applied one at a time. Less is known of the consequences of the exposure of cells to combinations of neuropeptides. The possibility that CGRP and SP are required together to affect PTH secretion was investigated. Incubating the primary cell cultures with CGRP and SP simultaneously at 1.25 mM Ca^{++} did not affect PTH secretion for up to 60 min.

The cultures were responsive to calcium, secreting a significantly greater amount of PTH in response to 0.5 mM Ca^{++} than to 2.0 mM Ca^{++} (Table 3.2). This is consistent with the response of parathyroid chief cells to extracellular Ca^{++}. At high extracellular Ca^{++} concentrations, the cultures continued to show a non-suppressible component of PTH secretion, which is also consistent with the normal physiological response of these cells to high calcium levels.

Isoproterenol and dopamine, which have been well documented to stimulate PTH secretion (Brown *et al.* 1976, 1977a, 1977b, 1977c, 1983; Hanley and Wellings 1985), were also found to significantly increase PTH secretion from the cell cultures (Table 3.2). As the cultured parathyroid cells responded normally to isoproterenol and dopamine, it is likely that they have replaced or have not lost their cell surface receptors as a result of the digestion procedure.

I do not feel it is likely that the conditions of the incubations caused degeneration of CGRP or SP. Endopeptidases capable of degrading CGRP and SP have been isolated from cerebrospinal fluid (Le Grevès *et al.* 1989; Nyberg *et al.* 1984), but such peptidases have not been identified in serum, and it is unlikely that CGRP and SP were being acted upon by these peptidases within the culture incubations. Furthermore, Brain and Williams (1985) and Brain *et al.* (1986b) reported that CGRP retains its biological activity upon incubation in human blood or plasma for 1 h at 37°C. In contrast, incubating SP in rat plasma for 1 h at 37°C resulted in a loss of 89% biological activity (Lembeck *et al.* 1978). With the concentration of FCS used in the cultures, any enzymes present probably would have had little inactivating effect on SP.

The CGRP and SP used in these studies are synthetic peptides, and there is no assurance that their effects truly mirror physiological events. The possibility that these neuropeptides must be further cleaved, or processed to express their biological activity cannot be ruled out. However, other investigators have found

synthetic CGRP and SP active in many *in vitro* cell systems. Although I was unable to demonstrate any effect of CGRP or SP on PTH secretion, a possible role for these neuropeptides on PTH secretion *in vivo* cannot be ruled out. The compound may have been given in doses or over time periods different from usual *in vivo* circumstances. Some studies have found PTH undergoes circadian or pulsatile variations. The neuropeptides may have to be released in a pulsatile manner to have an effect on PTH secretion.

4.4 POSSIBLE ROLES FOR CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P IN THE BOVINE PARATHYROID GLAND

The above evidence suggests that CGRP and SP may not play a direct role in PTH secretion. The distribution of the fibres within the bovine parathyroid gland suggests other possible functions for these neuropeptides. CGRP and SP found co-localized to fibres within the vasculature of the parathyroid gland suggests a likely vasomotor role for these sensory nerves. Brain *et al.* (1985) reported a direct vasodilatory effect of CGRP on aortic rings precontracted with norepinephrine. CGRP is a potent dilator of human and porcine coronary vessels (Greenwald *et al.* 1986; McEwan *et al.* 1986). Dilatation of skin arterioles of man and rabbit following intradermal injection, and dilatation of the hamster cheek pouch after topical application have also been reported (Brain *et al.* 1985). αCGRP and βCGRP appear to be equipotent as vasodilators (Brain *et al.* 1986b).

This may explain the presence of the peptidergic fibres near the tunica media of arteries and its absence from the veins. CGRP and SP, released from the peptidergic fibres, may diffuse into the tunica media causing relaxation of the vascular smooth muscles.

The physiological mode of vasodilation is not fully understood. It is not clear as to whether CGRP is endothelial requiring, and may depend on the location of the vessel. Endothelial-dependent relaxation has been found in rat aorta (Brain *et al.* 1985), but relaxation is independent of endothelium in certain rat, rabbit, and cat arteries (Edvinsson *et al.* 1985; Hanko *et al.* 1985). Sigrist *et al.* (1986) identified binding of CGRP to the tunica adventitia and tunica media in rat aorta. CGRP does not cause protein extravasation (Brain and Williams 1985; Brain *et al.* 1985, 1986b; Kubota *et al.* 1985).

In the case of endocrine glands innervated with SP, the release of SP may increase the rate at which the hormone enters the circulation from the extracellular space. Although SP is 1000-times less potent than CGRP as a vasodilator in subcutaneous vessels (Brain *et al.* 1985, 1986b), it has recently been demonstrated that SP increases protein extravasation (De Sanctis *et al.* 1990; Prior *et al.* 1990), probably through the release of histamine from mast cells (Fewtrell *et al.* 1982; Foreman *et al.* 1983). SP has also been found to require an intact endothelium in renal, celiac, and superior mesenteric arteries from cat, dog, and rabbit (Edvinsson *et al.* 1985; Furchgott *et al.* 1983; Zawadzki

et al. 1981).

There are data that suggest CGRP and SP play a role in neurogenic inflammation. It is possible that CGRP- and SP-LI nerves in the parathyroid are involved in this inflammatory response. CGRP- and SP-LI nerves have been identified in close proximity to mast cells in the rat (Domeij *et al.* 1991), and often make contact with them (Crivellato *et al.* 1991; Stead *et al.* 1987). Levine (1928) reported the presence of large numbers of mast cells in the bovine parathyroid gland, while Capen *et al.* (1965) and Zawistowski (1966) found only the occasional mast cell within the stroma of the gland. It is likely that fibres within the bovine parathyroid gland which deviate from the vasculature are associated with stromal mast cells. This would fit into the idea that these peptidergic fibres are involved in neurogenic inflammation via an axon reflex. The release of CGRP within the arterial walls could induce vasodilation, while SP release from the same fibres could be responsible for plasma extravasation by inducing mast cells to release histamine. The resultant plasma extravasation may aid in the movement of PTH from the extracellular fluid into the circulation, while the vasodilation could increase parathyroid blood flow, which would enhance the rate at which PTH enters the general circulation. At the same time, these potential actions of CGRP and SP on the parathyroid gland would increase the rate at which possible secretagogues in the circulation gain access to the parenchymal cells.

The notion of peptidergic fibres causing inflammation in the parathyroid gland maybe important in looking for an indirect affect of CGRP and SP on PTH secretion. The release of histamine by mast cells in response to CGRP or SP may have an effect on PTH secretion. Several groups have found that histamine causes an increase in parathyroid cell cAMP levels, and stimulates PTH secretion *in vitro* (Abboud *et al.* 1981; Brown 1980; Williams *et al.* 1981) and *in vivo* (Williams *et al.* 1981). The investigators found the histamine effects could be blocked by the H_2-receptor antagonist cimetidine. Bovine mast cells have also been reported to contain high levels of dopamine (Bertler *et al.* 1959), a known PTH secretagogue. It is conceivable that CGRP and SP may indirectly stimulate PTH secretion by causing mast cells to release histamine and/or dopamine during an inflammatory response. The cell cultures utilized in this study would have eliminated the required association between parathyroid and mast cells.

4.5 A ROLE FOR CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P IN CALCIUM HOMEOSTASIS

Despite the above evidence that CGRP lacks an effect on PTH secretion, at least over 90 min *in vitro*, there is a growing amount of evidence suggesting that CGRP can modulate calcium metabolism. However, these effects are species-specific. In rats, most of the data have demonstrated that CGRP has hypocalcemic properties similar to calcitonin *in vitro* and *in vivo*. The i.v. injection

of CGRP causes a dose-dependent decrease in total plasma calcium (Bevis *et al.* 1987; Datta *et al.* 1989; Tippins *et al.* 1984). Zaidi *et al.* (1988) reported a significant reduction in total plasma calcium levels after 60 min infusion, or 30 min following the intramuscular, i.p., i.v., or subcutaneous injection of hCGRP, which had a duration of 1 to 2 h.

The majority of evidence suggests that CGRP's hypocalcemic properties in rats are mediated by interacting with the skeleton. Goltzman and Mitchell (1985) were the first to demonstrate the binding of CGRP to bone. Roos *et al.* (1986) found that CGRP inhibits the release of ^{45}Ca from organ cultures and lowers blood calcium levels. CGRP was also able to cause an escape phenomenon (Roos *et al.* 1986; Yamamoto *et al.* 1986) similar to that seen with prolonged calcitonin exposure (Wener *et al.* 1972).

It is likely that CGRP's hypocalcemic properties are mediated via inactivation of osteoclasts. In support of this notion, Zaidi *et al.* (1987a, 1987b, 1988) found CGRP directly inhibited bone resorption from isolated osteoclasts. However, the dose of hCGRP or rCGRP required for maximal hypocalcemic effects are consistently 100-1000 fold greater than that required for calcitonin (Bevis *et al.* 1987; D'Souza *et al.* 1986; Miyaura *et al.* 1992; Roos *et al.* 1986; Tippins *et al.* 1984; Yamamoto *et al.* 1986; Zaidi *et al.* 1987a, 1987b, 1988). It has been suggested that CGRP's ability to modulate calcium levels is due to a weakly agonistic action on the calcitonin receptor. Even though salmon calcitonin and

CGRP are only 30% homologous (Breimer *et al.* 1988), their size and charge are similar, and they share amino-terminal cysteine rings and carboxy-terminal amidated residues, which might result in similar conformational structures of these two peptides such that they have affinities for the same receptors. Goltzman and Mitchell (1985) found CGRP and calcitonin cross react with each others receptors, but with lower affinity to the other's receptor in many tissues, including bone.

There is less consistent evidence in other species for a role of CGRP in modulating calcium homeostasis. Joborn *et al.* (1991) found no difference in PTH secretion from dispersed bovine parathyroid cell suspensions at 1.25 mM Ca^{++} during 30 minute exposures to 10^{-7} M or 10^{-6} M CGRP. In the rabbit and chicken, the effects of CGRP somewhat resemble those of PTH. In rabbits, 5 μg/kg calcitonin and CGRP were equipotent in their hypocalcemic effects (Bevis *et al.* 1987; Tippins *et al.* 1984). However, increasing the dose of CGRP to 10 μg/kg resulted in the initial hypocalcemia being succeeded by hypercalcemia. Bevis *et al.* (1990) found the i.v. injection of CGRP into chickens also caused an initial 15 min hypocalcemia which was succeeded by hypercalcemia 30 min after the CGRP injection. Ancill *et al.* (1990) found no change in calcium levels 20 min after CGRP injection into chickens, but reported a marked hyercalcemia at 60 min. Ancill *et al.* (1991) also found that CGRP caused a high level of calcium uptake into bone *in vivo*, which had a duration

of 10 min, as demonstrated by an elevation into a variety of bones of a simultaneous administered ^{45}Ca label. This transient uptake of calcium could explain the initial hypocalcemic response by CGRP in chickens reported by Bevis *et al.* (1990).

Many investigators have been able to isolate populations of bone cells responsive to CGRP but not to calcitonin, which might explain the PTH-like effects of CGRP found in rabbits and chickens. Crawford *et al.* (1986) found that 2×10^{-9} M CGRP increased cAMP production 30-40 fold over controls in cultured osteoblast-like cells. In an osteogenic sarcoma subclone (UMR 106-01) with no measurable calcitonin receptors or response, CGRP caused a dose-dependent increase in cAMP production (Michelangeli *et al.* 1986). Michelangeli *et al.* (1989) found production of cAMP by CGRP and PTH were additive in mouse, rat, and chicken osteoblast-rich bone cell cultures. Furthermore, PTH inhibitors had no effect on the response to CGRP. This suggests that bone contains a subpopulation of osteoblast-like cells with CGRP receptors linked to adenylate cyclase. However, heterogeneity of the cells cannot be ruled out, and the exact identification of the cells is unknown. In the subclones, the expression of CGRP receptors maybe unrelated to the osteoblastic phenotype of those cells.

The role of CGRP in osteoblast function is unknown, as the circulating levels of CGRP are too low to have an effect on bone mineralization, and Crawford *et al.* (1986) found CGRP had no effect on osteocalcin or prostaglandin

production by cultured osteoblast-like cells.

There has been very little investigation of an effect of SP on calcium homeostasis. Zaidi *et al.* (1988) found the i.v. injection of 500 pmol SP into rats had no effect on total plasma calcium levels. Joborn *et al.* (1991) also found no difference in PTH secretion from bovine parathyroid cell suspensions at 1.25 mM Ca^{++} during 30 minute incubation with SP at concentrations between 10^{-9} M to 10^{-5} M.

The possibility that CGRP and SP are able to effect calcium homeostasis is further complicated by the finding of CGRP- and SP-LI nerve fibres within the skeleton. SP has been localized by immunohistochemistry to nerve fibres within the porcine periosteum (Hohmann *et al.* 1986), while CGRP- and SP-LI nerves have also been identified in the periosteum of rat mandible, tibia, and calvarium (Hill and Elde 1988, 1991). CGRP- and SP-containing nerves have also been identified within the rat tibia (Bjurholm *et al.* 1988, 1889; Hill and Elde, 1991). Many of the fibres were found associated with the vasculature. However, several fibres deviated from the vasculature and terminated in networks of beaded varicosities near the bone surface. This suggests a role for these neuropeptides in the periosteum separate from vasodilation. As neither CGRP- or SP-containing fibres could be demonstrated between bone lamellae, it appears unlikely that their distribution is suitable for a role in calcium homeostasis. However, Bjurholm *et al.* (1988) reported the greatest density of fibres at the epiphyseal plate, which

suggests that these fibres may have an important role in growth at the epiphyseal plate.

Although there is little evidence for a role of CGRP- or SP-containing fibres in calcium metabolism, capsaicin-treated rats showed a dramatic reduction in CGRP- and SP-LI nerve fibres in the periosteum (Hill and Elde, 1991), suggesting these fibres are primary afferent in origin. The release of neuropeptides from unmyelinated C fibres may play a primary role in mechanoreception or nociception within bone. There is also a growing body of evidence that suggests these fibres may not only be involved in the perception of pain, but may also contribute to the healing process. Bernard and Shih (1990) and Shih and Wang (1992) found that CGRP had an osteogenic effect, increasing the number and size of bone colonies *in vitro*. Sensory nerve impulses caused by bone injury may release CGRP and SP by axon reflex into the vicinity of the osteoprogenitor cells, which maybe important for growth during healing.

Reimann and Christensen (1977) found a greater number of nerves in osteoarthritic than healthy human bone. CGRP was found to be high in human osteoarthritic tibial periosteum (Grönblad *et al.* 1984). Kvinnsland and Heyeraas (1992) and Taylor *et al.* (1988) found an increase in CGRP and SP fibres in rat molars in response to injury. These findings suggest that CGRP and SP may play a role in healing of injured bone, and that nerve proliferation may be required to deliver the neuropeptides in high enough concentration in close

proximity to the site of injury to promote growth and possibly mineralization of new bone during the healing process.

It is also of some interest that PAS-57, the amino-terminal cleavage peptide of procalcitonin, which has been identified within the circulation at levels 1.7 times greater than calcitonin, recently has been reported to have mitogenic properties on osteoblast rich cultures at nanomolar concentrations *in vitro* (Burns *et al.* 1989). PAQ-55, the amino-terminal peptide derived from cleavage of proCGRP has its first 50 amino acid residues identical to PAS-57. Although its presence has not been confirmed yet, it is conceivable PAQ-55 maybe released from osseous nerve fibres in close proximity to bone cells to play a physiological role on bone mineralization.

4.6 FUTURE STUDIES

4.6.1 What are the Origins of the Neuropeptide Fibres?

This study did not attempt to characterize the origin of the CGRP and SP fibres within the bovine parathyroid gland. Because it is well documented that CGRP and SP are present in primary sensory C fibres, it is likely that this is the case in the bovine parathyroid glands. Depletion of neuropeptides from these fibres using capsaicin or its more potent analog resiniferatoxin, would confirm the primary sensory origin of these peptidergic fibres. The location of the parathyroid glands suggests that their sensory fibres would be located in the

trigeminal or nodose ganglia. Injection of the tracers horseradish peroxidase or lucifer yellow into the parathyroid glands and locating the labelled sensory cell bodies would identify the source of these fibres.

4.6.2 Are other Neuropeptides Present in the Parathyroid Gland?

1) Studies to further characterize neuropeptides in the bovine parathyroid gland should be carried out. As several mammalian tachykinins have been identified in nervous tissue, immunohistochemical techniques using antisera specific for the different tachykinins should be utilized to determine more precisely the relative abundance of the various tachykinins within the parathyroid gland. Radio-immunoassays specific for the different tachykinins could also be utilized to determine the presence and relative abundance of the various tachykinins within the parathyroid gland.

2) There is a complex distribution of neuropeptides in the nervous system. In addition to CGRP, SP has been found co-localized with arginine vasopressin (Kai-Kai *et al.* 1986), bombesin (Fuxe *et al.* 1983), CCK (Dalsgaard *et al.* 1982; Leah *et al.* 1985; Tuchscherer and Seybold 1985), NKA (Dalsgaard *et al.* 1985), and VIP (Leah *et al.* 1985) within primary sensory neurons. CGRP has also been co-localized with NKA (Diez Guerra *et al.* 1988) in these fibres. The possibility that other neuropeptides are present in the parathyroid innervation and have physiological effects on the parathyroid gland should also be investigated. Joborn *et al.* (1991) found VIP increased PTH secretion from bovine parathyroid

glands. The i.v. injection of CGRP (3.0 nmol) into mice enhances the stimulatory effect of VIP on thyroxine secretion (Grunditz *et al.* 1986). A similar phenomenon may take place in the parathyroid gland whereby CGRP enhances VIP stimulated PTH secretion.

4.6.3 What are the Roles of Calcitonin Gene-Relate Peptide and Substance P in the Parathyroid Gland?

There is a possibility that CGRP and SP are able to indirectly effect PTH secretion as a result of their potential role in neurogenic inflammation. During the inflammatory response, histamine and/or dopamine released from mast cells near the parenchyma might reach high enough concentrations to stimulate PTH secretion. The perifusion of small pieces of gland (Hanley *et al.* 1980), which contain stromal components and intact mast cells in close anatomical orientation to the parenchyma could be used to investigate this possibility. The ability of histamine and dopamine antagonists to block any effects of PTH secretion would suggest that histamine or dopamine are released from within the tissue, supporting the involvement of the mast cells.

However, CGRP and SP in the bovine parathyroid gland may play a role in nociception and neurogenic inflammation and have no effect on PTH secretion. The natural nociceptive stimuli for these afferent fibres in the parathyroid gland are unknown at this time. SP is a mitogen for fibroblasts (Nilsson *et al.* 1985), T-lymphocytes (Payan *et al.* 1983; Stanisz *et al.* 1986), and smooth muscle cells

(Mitsuhashi and Payan 1987; Payan 1985), which could play an important role in the growth of tissue in response to being damaged. Therefore, SP may act as a mitogen on parathyroid tissue. The release of neuropeptides from fibres in abnormal conditions could contribute to hyperplasia of the parathyroid gland. The possibility that SP or CGRP cause parathyroid growth or hyperplasia could be tested by using [^{3}H]thymidine incorporation into parathyroid cultures as an index of DNA synthesis and cell proliferation.

These studies would add to our knowledge of the distribution and effects of neuropeptides in the endocrine system. The more we know about the distribution and effects of neuropeptides in the parathyroid gland, the closer we can come to understanding the role of the parathyroid gland in health and disease. This increased knowledge could enable improved treatment of parathyroid disorders.

SUMMARY

1) Using indirect immunohistochemistry, I was able to identify human and rat CGRP-LI nerve fibres in the bovine parathyroid gland within the stroma, arterial walls, and surrounding parenchymal lobules. However, no CGRP-LI fibres were found to enter the parenchyma and synapse with the chief cells.

2) SP-LI fibres were also identified within the stroma, arterial walls, and encircling the parenchyma, with a staining intensity and distribution comparable to those for CGRP.

3) Fibres containing both CGRP- and SP-LI were observed in double-staining experiments. All of the fibres showed identical staining for both CGRP and SP.

4) Primary bovine parathyroid cell cultures incubated for up to 90 min with either CGRP or SP had no direct effect on PTH secretion at concentrations between 10^{-8} M to 10^{-5} M at normal physiological concentrations of calcium (1.25 mM Ca^{++}).

5) In the presence of either low (0.5 mM) or high (2.0 mM) extracellular concentrations of Ca^{++}, CGRP or SP did not significantly affect the amount of PTH secreted over control values from the cultures for incubation times as long as 60 min.

6) Cultures incubated for up to 60 min with 10^{-10} M to 10^{-6} M CGRP and SP together, in the presence of 1.25 mM Ca^{++}, had no significant effect on PTH secretion.

LITERATURE CITED

Abboud HE, Zimmerman D, Edis AJ, Heath III H, Dousa TP (1981) Histamine and human parathyroid adenoma: effect on adenosine 3′,5′-monophosphate *in vitro*. *J Clin Endocrinol Metab* **53**:276-281

Abe M, Sherwood LM (1972) Regulation of parathyroid hormone secretion by adenyl cyclase. *Biochem Biophys Res Commun* **48**:396-401

Ahrén B, Aluments J, Ericsson M, Fahrenkrug J, Fahrenkrug L, Håkanson R, Hedner P, Lorén I, Melander A, Rerup C, Sundler F (1980) VIP occurs in intrathyroidal nerves and stimulates thyroid hormone secretion. *Nature* **287**:343-345

Ahrén B, Grunditz T, Ekman R, Håkanson R, Sundler F, Uddman R (1983) Neuropeptides in the thyroid gland: distribution of substance P and gastrin/cholecystokinin and their effects on the secretion of iodothyronine and calcitonin. *Endocrinology* **113**:379-384

Alevizaki M, Shiraishi A, Rassool FV, Ferrier GJM, MacIntrye I, Legon S (1986) The calcitonin-like sequence of the βCGRP gene. *FEBS Lett* **206**:47-52

Altenähr E (1971) Electron microscopical evidence for innervation of chief cells in human parathyroid gland. *Experientia* **27**:1077

Amara SG, Arriza JL, Leff SE, Swanson LW, Evans RM, Rosenfeld MG (1985) Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. *Science* **229**:1094-1097

Amara SG, Jones V, Rosenfeld MG, Ong ES, Evans RM (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* **298**:240-245

Ancill AK, Bascal ZA Whitaker G, Dacke CG (1990) Effects of rat and chicken calcitonin gene-related peptides (CGRP) upon calcium metabolism in chicks. *Regul Pept* **30**:231-238

Ancill AK, Bascal ZA Whitaker G, Dacke CG (1991) Calcitonin gene-related peptide promotes transient radiocalcium uptake into chick bone *in vivo*. *Exp Physiol* **76**:143-146

Arnaud CD, Tsao HS, Littledike T, Hess J, Laakso K, Bischoff J (1971) Radioimmunoassay of human parathyroid hormone in serum. *J Clin Invest* **50**:21-34

Atwal OS (1981) Myelinated nerve fibers in the parathyroid gland of the dog: a light and electron-microscopic study. *Acta Anat (Basel)* **109**:3-12

Barja F, Mathison R, Huggel H (1983) Substance P-containing nerve fibres in large peripheral blood vessels of the rat. *Cell Tissue Res* **229**:411-422

Bar-Shavit Z, Goldman R, Stabinsky Y, Gottlieb P, Fridkin M, Teichberg VI, Blumberg S (1980) Enhancement of phagocytosis - a newly found activity of substance P residing in its N-terminal tetrapeptide sequence. *Biochem Biophys Res Commun* **94**:1445-1451

Bayliss WM (1901) On the origin from the spinal cord of the vaso-dilator fibres of the hind-limb, and on the nature of these fibres. *J Physiol (Lond)* **26**:173-209

Bernard GW, Shih C (1990) The osteogenic stimulating effect of neuroactive calcitonin gene-related peptide. *Peptides* **11**:625-632

Berridge MJ (1984) Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J* **220**:345-360

Bertler Å, Falck B, Hillarp N-Å, Rosengren E, Torp A (1959) Dopamine and chromaffin cells. *Acta Physiol Scand* **47**:251-258

Bevan S, Szolcsányi J (1990) Sensory neuron-specific actions of capsaicin: mechanisms and applications. *Trends Pharmacol Sci* **11**:330-333

Bevis PJR, Lynch C, Beacham J, Girgis SI, Morris H, Azria M (1987) The plasma calcium modulating effects of calcitonin gene-related peptide. In: Cohn DV, Martin TJ, Meunier PJ (eds) Calcium Regulation and Bone Metabolism: Basic and Clinical Aspects Vol 9. Elsevier Science Publishers B.V., Amsterdam, p 567 (Abst.)

Bevis PJR, MacIntyre I, Zaidi M (1986) Further evidence for the neural release of plasma calcitonin gene-related peptide. *Br J Pharmacol* **88 (Suppl)**:314P (Abst.)

Bevis PJR, Zaidi M, MacIntyre I (1990) A dual effect of calcitonin gene-related peptide on plasma calcium levels in the chick. *Biochem Biophys Res Commun* **169**:846-850

Bishop AE, Polak JM, Green IC, Bryant MG, Bloom SR (1980) The location of VIP in the pancreas of man and rat. *Diabetologia* **18**:73-78

Bjurholm A, Kreicbergs A, Brodin E, Schultzberg M (1988) Substance P- and CGRP-immunoreactive nerves in bone. *Peptides* **9**:165-171

Bjurholm A, Kreicbergs A, Schultzberg M (1989) Fixation and demineralization of bone tissue for immunohistochemical staining of neuropeptides. *Calcif Tissue Int* **45**:227-231

Blum JW, Guillebeau A, Binswanger U, Kunz P, Da Prada M, Fischer JA (1978) Effects of alpha-adrenergic stimulation and blockage on plasma parathyroid hormone concentrations in cows. *Acta Endocrinol (Copenh)* **88**:535-544

Brain SD, MacIntyre I, Williams TJ (1986a) A second form of human calcitonin gene-related peptide which is a potent vasodilator. *Eur J Pharmacol* **124**:349-352

Brain SD, Tippins JR, Morris HR, MacIntyre I, Williams TJ (1986b) Potent vasodilator activity of calcitonin gene-related peptide in human skin. *J Invest Dermatol* **87**:533-536

Brain SD, Williams TJ (1985) Inflammatory oedema induced by synergism between calcitonin gene-related peptide (CGRP) and mediators of increased vascular permeability. *Br J Pharmacol* **86**:855-860

Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I (1985) Calcitonin gene-related peptide is a potent vasodilator. *Nature* **313**:54-56

Breimer LH, MacIntyre I, Zaidi M (1988) Peptides from the calcitonin genes: molecular genetics, structure and function. *Biochem J* **255**:377-390

Breslau NA (1988) Calcium homeostasis. In: Griffin JE, Ojeda SR (eds) Textbook of endocrine physiology. Oxford University Press, New York, pp 273-275

Brimijoin S, Lundberg JM, Brodin E, Hökfelt T, Nilsson G (1980) Axonal transport of substance P in the vagus and sciatic nerves of the guinea pig. *Brain Res* **191**:443-457

Brown EM (1980) Histamine receptors on dispersed parathyroid cells from pathological human parathyroid tissue. *J Clin Endocrinol Metab* **51**:1325-1329

Brown EM, Carroll RJ, Aurbach GD (1977a) Dopaminergic stimulation of cyclic AMP accumulation and parathyroid hormone release from dispersed bovine parathyroid cells. *Proc Natl Acad Sci USA* **74**:4210-4213

Brown EM, Gardner DG, Windeck RA, Aurbach GD (1978a) Relationship of intracellular 3′,5′-adenosine monophosphate accumulation to parathyroid hormone release from dispersed bovine parathyroid cells. *Endocrinology* **103**:2323-2333

Brown EM, Hurwitz S, Aurbach GD (1976) Preparation of viable isolated bovine parathyroid cells. *Endocrinology* **99**:1582-1588

Brown EM, Hurwitz S, Aurbach GD (1977b) Beta-adrenergic stimulation of cyclic AMP content and parathyroid hormone release from isolated bovine parathyroid cells. *Endocrinology* **100**:1696-1702

Brown EM, Hurwitz SH, Aurbach GD (1978b) α-Adrenergic inhibition of adenosine 3′,5′-monophosphate accumulation and parathyroid hormone release from dispersed bovine parathyroid cells. *Endocrinology* **103**:893-899

Brown EM, Hurwitz S, Woodard CJ, Aurbach GD (1977c) Direct identification of beta-adrenergic receptors on isolated bovine parathyroid cells. *Endocrinology* **100**:1703-1709

Brown EM, Watson EJ, Leombruno R, Underwood RH (1983) Extracellular calcium is not necessary for acute, low calcium- or dopamine-stimulated PTH secretion in dispersed bovine parathyroid cells. *Metabolism* **32**:1038-1044

Buffa R, Capella C, Solcia E, Frigerio B, Said SI (1977) Vasoactive intestinal peptide (VIP) cells in the pancreas and gastro-intestinal mucosa. *Histochemistry* **50**:217-227

Burns DM, Forstrom JM, Friday KE, Howard GA, Roos BA (1989) Procalcitonin's amino-terminal cleavage peptide is a bone-cell mitogen. *Proc Natl Acad Sci USA* **86**:9519-9523

Capen CC, Koestner A, Cole CR (1965) The ultrastructure and histochemistry of normal parathyroid glands of pregnant and nonpregnant cows. *Lab Invest* **14**:1673-1690

Cardinali DP, Ladizesky MG (1985) Changes in parathyroid hormone and calcium levels after superior cervical ganglionectomy of rats. *Neuroendocrinology* **40**:291-296

Care AD, Bruce JB, Boelkins J, Kenny AD, Conaway H, Anast CS (1971) Role of pancreozymin-cholecystokinin and structurally related compounds as calcitonin secretogogues. *Endocrinology* **89**:262-271

Carlton SM, McNeill DL, Chung K, Coggeshall RE (1987) A light and electron microscopic level analysis of calcitonin gene-related peptide (CGRP) in the spinal cord of the primate: an immunohistochemical study. *Neurosci Lett* **82**:145-150

Chambers TJ, Chambers JC, Symonds J, Darby JA (1986) The effect of human calcitonin on the cytoplasmic spreading of rat osteoclasts. *J Clin Endocrinol Metab* **63**:1080-1085

Chambers TJ, Magnus CJ (1982) Calcitonin alters behaviour of isolated osteoclasts. *J Pathol* **136**:27-39

Chambers TJ, McSheehy PMJ, Thompson BM, Fuller K (1985) The effect of calcium-regulating hormones and prostaglandins on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. *Endocrinology* **116**:234-239

Chambers TJ, Moore A (1983) The sensitivity of isolated osteoclasts to morphological transformation by calcitonin. *J Clin Endocrinol Metab* **57**:819-824

Chambers TJ, Revell PA, Fuller K, Athanasou NA (1984) Resorption of bone by isolated rabbit osteoclasts. *J Cell Sci* **66**:383-399

Chang MM, Leeman SE, Niall HD (1971) Amino acid sequence of substance P. *Nature (New Biol)* **232**:86-87

Clark A, Lewis CE, Willis AC, Cooper GJS, Morris JF, Reid KBM, Terner RC (1987) Islet amyloid formed from diabetes-associated peptide may be pathogenic in type-2 diabetes. *Lancet* **2**:231-234

Coons AH, Leduc EH, Connolly JM (1955) Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J Exp Med* **102**:49-60

Cooper GJS, Willis AC, Clark A, Turner RC, Sim RB, Reid KBM (1987) Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. *Proc Natl Acad Sci USA* **84**:8628-8632

Copp DH, Cameron EC, Cheney BA, Davidson AGF, Henze KG (1962) Evidence for calcitonin-a new hormone from the parathyroid that lowers blood calcium. *Endocrinology* **70**:638-649

Costa M, Cuello AC, Furness JB, Franco R (1980) Distribution of enteric neurons showing immunoreactivity for substance P in the guinea-pig ileum. *Neuroscience* **5**:323-331

Crawford A, Evans DB, Skjodt H, Bersford JN, MacIntyre I, Russell RGG (1986) Effect of human calcitonin gene-related peptide on human bone-derived cells in culture. *Bone* **7**:157-158 (Abst.)

Crivellato E, Damiani D, Mallardi F, Travan L (1991) Suggestive evidence for a microanatomical relationship between mast cells and nerve fibres containing substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide, and somatostatin in the rat mesentery. *Acta Anat (Basel)* **141**:127-131

Cuello AC, Galfre G, Milstein C (1979) Detection of substance P in the central nervous system by a monoclonal antibody. *Proc Natl Acad Sci USA* **76**:3532-3536

Dalsgaard C-J, Haegerstrand A, Theodorsson-Norheim E, Brodin E, Hökfelt T (1985) Neurokinin A-like immunoreactivity in rat primary sensory neurones; coexistence with substance P. *Histochemistry* **83**:37-39

Dalsgaard C-J, Vincent SR, Hökfelt T, Lundberg JM, Dahlström A, Schultzberg M, Dockray GJ, Cuello AC (1982) Coexistence of cholecystokinin- and substance P-like peptides in neurons of the dorsal root ganglia of the rat. *Neurosci Lett* **33**:159-163

Datta HK, Zaidi M, Wimalawansa SJ, Ghatei MA, Beacham JL, Bloom SR, MacIntyre I (1989) *In vivo* and *in vitro* effects of amylin and amylin-amide on calcium metabolism in the rat and rabbit. *Biochem Biophys Res Commun* **162**:876-881

De Sanctis GT, App EM, Trask JK, De Sanctis BI, Remmers JE, Green FHY, Man SFP, King M (1990) Resorptive clearance and transepithelial potential difference in capsaicin-treated F344 rats. *J Appl Physiol* **68**:1826-1832

Diez Guerra FJ, Zaidi M, Bevis P, MacIntyre I, Emson PC (1988) Evidence for release of calcitonin gene-related peptide and neurokinin A from sensory nerve endings *in vivo*. *Neuroscience* **25**:839-846

Domeij S, Carlsöö B, Dahlqvist Å, Forsgren S (1991) Occurrence of mast cells in relation to the distribution of nerve fibers in the rat larynx. *Acta Otolaryngol (Stockh)* **111**:981-989

D'Souza SM, MacIntyre I, Girgis SI, Mundy GR (1986) Human synthetic calcitonin gene-related peptide inhibits bone resorption *in vitro*. *Endocrinology* **119**:58-61

Duckles SP, Buck SH (1982) Substance P in the cerebral vasculature: depletion by capsaicin suggests a sensory role. *Brain Res* **245**:171-174

Duckles SP Levitt B (1984) Specificity of capsaicin treatment in the cerebral vasculature. *Brain Res* **308**:141-144

Edvinsson L, Fredholm BB, Hamel E, Jansen I, Verrecchia C (1985) Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat. *Neurosci Lett* **58**:213-217

Edvinsson L, Uddman R (1982) Immunohistochemical localization and dilatatory effect of substance P on human cerebral vessels. *Brain Res* **232**:466-471

Emson PC, Zaidi M (1989) Further evidence for the origin of circulating calcitonin gene-related peptide in the rat. *J Physiol (Lond)* **412**:297-308

Fewtrell CMS, Foreman JC, Jordan CC, Oehme P, Renner H, Stewart JM (1982) The effects of substance P on histamine and 5-hydroxytryptamine release in the rat. *J Physiol (Lond)* **330**:393-411

Fischer JA, Blum JW, Binswanger U (1973) Acute parathyroid hormone response to epinephrine in vivo. *J Clin Invest* **52**:2434-2440

Fisher LA, Kikkawa DO, Rivier JE, Amara SG, Evans RM, Rosenfeld MG, Vale WW, Brown MR (1983) Stimulation of noradrenergic sympathetic outflow by calcitonin gene-related peptide. *Nature* **305**:534-536

Foreman JC, Jordan CC, Oehme P, Renner H (1983) Structure-activity relationships for some substance P-related peptides that cause wheal and flare reactions in human skin. *J Physiol (Lond)* **335**:449-465

Foster GV (1968) Calcitonin (thyrocalcitonin). *N Engl J Med* **279**:349-360

Foster GV, MacIntrye I, Pearce AGE (1964) Calcitonin production and the mitochondrion-rich cells of the dog thyroid. *Nature* **203**:1029-1030

Fox J, Offord KP, Hunter III H (1981) Episodic secretion of parathyroid hormone in the dog. *Am J Physiol* **241**:E171-E177

Franco-Cereceda A, Henke H, Lundberg JM, Petermann JB, Hökfelt T, Fischer JA (1987) Calcitonin gene-related peptide (CGRP) in capsaicin-sensitive substance P-immunoreactive sensory neurons in animals and man: distribution and release by capsaicin. *Peptides* **8**:399-410

Furchgott RF, Cherry PD, Zawadzki JV (1983) Endothelium-dependent relaxation of arteries by acetylcholine, bradykinin, and other agents. In: Bevan JA, Fujiwara M, Maxwell RA, Mohri K, Shibata S, Toda N (eds) Vascular neuroeffector mechanisms: 4th international symposium. Raven Press, New York, pp 154-155

Furness JB, Papka RE, Della NG, Costa M, Eskay RL (1982) Substance P-like immunoreactivity in nerves associated with the vascular system of guinea-pigs. *Neuroscience* **7**:447-459

Fuxe K, Agnati LF, McDonald T, Locatelli V, Hökfelt T, Dalsgaard C-J, Battistini N, Yanaihara N, Mutt V, Cuello AC (1983) Immunohistochemical indications of gastrin releasing peptide - bombesin-like immunoreactivity in the nervous system of the rat. Codistribution with substance P-like immunoreactive nerve terminal systems and coexistence with substance P-like immunoreactivity in dorsal root ganglion cell bodies. *Neurosci Lett* **37**:17-22

Gamse R, Saria A (1985) Potentiation of tachykinin-induced plasma protein extravasation by calcitonin gene-related peptide. *Eur J Pharmacol* **114**:61-66

Gibbins IL, Furness JB, Costa M, MacIntyre I, Hillyard CJ, Girgis S (1985) Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. *Neurosci Lett* **57**:125-130

Gibson SJ, Polak JM, Bloom SR, Sabate IM, Mulderry PM, Ghatei MA, McGregor GP, Morrison JFB, Kelly JS, Evans RM, Rosenfeld MG (1984) Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. *J Neurosci* **4**:3101-3111

Girgis SI, Stevenson JC, Lynch C, Self CH, MacDonald DWR, Bevis PJR, Wimalawansa SJ, Morris HR, MacIntyre I (1985) Calcitonin gene-related peptide: potent vasodilator and major product of calcitonin gene. *Lancet* **2**:14-16

Gkonos PJ, Born W, Jones BN, Petermann JB, Keutmann HT, Birnbaum RS, Fischer JA, Roos BA (1986) Biosynthesis of calcitonin gene-related peptide and calcitonin by a human medullary thyroid carcinoma cell line. *J Biol Chem* **261**:14386-14391

Goltzman D, Mitchell J (1985) Interaction of calcitonin and calcitonin gene-related peptide at receptor sites in target tissues. *Science* **227**:1343-1345

Greenwald SE, Lever MJ, MacIntyre I, Morris HR, Tippins JR (1986) Human calcitonin gene-related peptide is a potent vasodilator in the pig coronary circulation. *Br J Pharmacol* **87 (Suppl)**:56P (Abst.)

Grönblad M, Liesi P, Korkala O, Karaharju E, Polak J (1984) Innervation of human bone periosteum by peptidergic nerves. *Anat Rec* **209**:297-299

Gross M, Kumar R (1990) Physiology and biochemistry of vitamin D-dependent calcium binding proteins. *Am J Physiol* **259**:F195-F209

Grunditz T, Ekman R, Håkanson R, Rerup C, Sundler F, Uddman R (1986) Calcitonin gene-related peptide in thyroid nerve fibers and C cells: effects on thyroid hormone secretion and response to hypercalcemia. *Endocrinology* **119**:2313-2324

Gulbenkian S, Merighi A, Wharton J, Varndell IM, Polak JM (1986) Ultrastructural evidence for the coexistence of calcitonin gene-related peptide and substance P in secretory vesicles of peripheral nerves in the guinea pig. *J Neurocytol* **15**:535-542

Guyton AC (1991a) Hemostasis and blood coagulation. In: Wonsiewicz MJ (ed) Textbook of medical physiology, 8th Ed. W.B. Saunders Co., Philadelphia, p 395

Guyton AC (1991b) Parathyroid hormone, calcitonin, calcium and phosphate metabolism, vitamin D, bone, and teeth. In: Wonsiewicz MJ (ed) Textbook of medical physiology, 8th Ed. W.B. Saunders Co., Philadelphia, pp 875-876

Guyton AC (1991c) Somatic sensations: II. Pain, headache, and thermal sensations. In: Wonsiewicz MJ (ed) Textbook of medical physiology, 8th Ed. W.B. Saunders Co., Philadelphia, pp 485-486

Guyton AC (1991d) The nervous system: A. General principles and sensory physiology. In: Wonsiewicz MJ (ed) Textbook of medical physiology, 8th Ed. W.B. Saunders Co., Philadelphia, pp 485-486

Habener JF, Kemper B, Potts Jr. JT (1975) Calcium-dependent intracellular degradation of parathyroid hormone: a possible mechanism for the regulation of hormone stores. *Endocrinology* **97**:431-441

Hanko J, Hardebo JE, Kåhrström J, Owman C, Sundler F (1985) Calcitonin gene-related peptide is present in mammalian cerebrovascular nerve fibres and dilates pial and peripheral arteries. *Neursci Lett* **57**:91-95

Hanley DA, Takatsuki K, Birnbaumer ME, Schneider AB, Sherwood LM (1980) In vitro perifusion for the study of parathyroid hormone secretion: effects of extracellular calcium concentration and beta-adrenergic regulation on bovine parathyroid hormone secretion in vitro. *Calcif Tissue Int* **32**:19-27

Hanley DA, Wellings PG (1985) Dopamine-stimulated parathyroid hormone release in vitro: further evidence for a two-pool model of parathyroid hormone secretion. *Can J Physiol Pharmacol* **63**:1139-1144

Hardebo JE, Suzuki N, Owman C (1989) Origins of substance P- and calcitonin gene-related peptide-containing nerves in the internal carotid artery of rat. *Neurosci Lett* **101**:39-45

Heath III H, Fox J, Fryer M, Laakso K, Schwegman C, Ryback K (1985) Electrical and chemical stimulation of cervical sympathetic nerves in the dog does not affect secretion of parathyroid hormone. *Endocrinology* **116**:1977-1982

Heath III H, Larson JM, Laakso K (1980) Provocative tests of parathyroid and C cell function in adrenalectomized and chemically sympathectomized rats. *Endocrinology* **107**:977-981

Heath III H, Sizemore GW (1977) Plasma calcitonin in normal man. Differences between men and women. *J Clin Invest* **60**:1135-1140

Heynen G, Franchimont P (1974) Human calcitonin radioimmunoassay in normal and pathological conditions. *Eur J Clin Invest* **4**:213-222

Hill EL, Elde R (1988) Calcitonin gene-related peptide-immunoreactive nerve fibers in mandibular periosteum of rat: evidence for primary afferent origin. *Neurosci Lett* **85**:172-178

Hill EL, Elde R (1991) Distribution of CGRP-, VIP-, DβH-, SP-, and NPY-immunoreactive nerves in the periosteum of the rat. *Cell Tissue Res* **264**:469-480

Hohmann EL, Elde RP, Rysavy JA, Einzig S, Gebhard RL (1986) Innervation of periosteum and bone by sympathetic vasoactive intestinal peptide-containing nerve fibers. *Science* **232**:868-871

Hökfelt T, Johansson O, Ljungdahl Å, Lundberg JM, Schultzberg M (1980) Peptidergic neurones. *Nature* **284**:515-521

Holman JJ, Craig RK, Marshall I (1986) Human α- and β-CGRP and rat α-CGRP are coronary vasodilators in the rat. *Peptides* **7**:231-235

Holzer P (1988) Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* **24**:739-768

Holzer P, Sametz W (1986) Capsaicin-sensitive afferent neurones involved in gastric mucosal protection. *Br J Pharmacol* **89 (Suppl)**:562P (Abst.)

Höppener JWM, Steenbergh PH, Zandberg J, Bakker E, Pearson PL, Geurts van Kessel AHM, Jansz HS, Lips CJM (1984) Localization of the polymorphic human calcitonin gene on chromosome 11. *Hum Genet* **66**:309-312

Höppener JWM, Steenbergh PH, Zandberg J, Geurts van Kessel AHM, Baylin SB, Nelkin BD, Jansz HS, Lips CJM (1985) The second human calcitonin/CGRP gene is located on chromosome 11. *Hum Genet* **70**:259-263

Hua X-Y, Theodorsson-Norheim E, Brodin E, Lundberg JM, Hökfelt T (1985) Multiple tachykinins (neurokinin A, neuropeptide K and substance P) in capsaicin-sensitive sensory neurons in the guinea-pig. *Regul Pept* **13**:1-19

Isono H, Shoumura S (1980) Effects of vagotomy on the ultrastructure of the parathyroid gland of the rabbit. *Acta Anat (Basel)* **108**:273-280

Jacobowitz DM, Brown EM (1980) Bovine parathyroid catecholamines: a chemical and histochemical study. *Experientia* **36**:115-116

Joborn H, Larsson R, Rastad J, Nygren P, Åkerström G, Ljunghall S (1991) Vasoactive intestinal polypeptide stimulates parathyroid hormone release by interaction with cyclic adenosine monophosphate production of bovine parathyroid cells. *Acta Endocrinol (Copenh)* **124**:54-59

Ju G, Hökfelt T, Brodin E, Fahrenkrug J, Fischer JA, Frey P, Elde RP, Brown JC (1987) Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. *Cell Tissue Res* **247**:417-431

Jubiz W, Canterbury JM, Reiss E, Tyler FH Frailey J, Bartholomew K, Creditor MA (1972) Circadian rhythm in serum parathyroid hormone concentration in human subjects: correlation with serum calcium, phosphate, albumin, and growth hormone levels. *J Clin Invest* **51**:2040-2046

Kage R, McGregor GP, Thim L, Conlon JM (1988) Neuropeptide-γ: a peptide isolated from rabbit intestine that is derived from γ-preprotachykinin. *J Neurochem* **50**:1412-1417

Kai-Kai MA, Anderton BH, Keen P (1986) A quantitative analysis of the interrelationships between subpopulations of rat sensory neurons containing arginine vasopressin or oxytocin and those containing substance P, fluoride-resistant acid phosphatase or neurofilament protein. *Neuroscience* **18**:475-486

Kangawa K, Minamino N, Fukuda A, Matsuo H (1983) Neuromedin K: a novel mammalian tachykinin identified in porcine spinal cord. *Biochem Biophys Res Commun* **114**:533-540

Knight DE, von Grafenstein H, Athayde CM (1989) Calcium-dependent and calcium-independent exocytosis. *Trends Neurosci* **12**:451-458

Krause JE, Hershey AD, Dykema PE, Takeda Y (1990) Molecular biological studies on the diversity of chemical signalling in tachykinin peptidergic neurons. *Ann N Y Acad Sci* **579**:254-272

Kripke DF, Lavie P, Parker D, Huey L, Deftos LJ (1978) Plasma parathyroid hormone and calcium are related to sleep stage cycles. *J Clin Endocrinol Metab* **47**:1021-1027

Kubota M, Moseley JM, Butera L, Dusting GJ, MacDonald PS, Martin TJ (1985) Calcitonin gene-related peptide stimulates cyclic AMP formation in rat aortic smooth muscle cells. *Biochem Biophys Res Commun* **132**:88-94

Kukreja SC, Hargis GK, Bowser EN, Henderson WJ, Fisherman EW, Williams GA (1975) Role of adrenergic stimuli in parathyroid hormone secretion in man. *J Clin Endocrinol Metab* **40**:478-481

Kvinnsland I, Heyeraas KJ (1992) Effect of traumatic occlusion on CGRP and SP immunoreactive nerve fibre morphology in rat molar pulp and periodontium. *Histochemistry* **97**:111-120

Larsson L-I, Fahrenkrug J, Holst JJ, Schaffalitzky de Muckadell OB (1978) Innervation of the pancreas by vasoactive intestinal polypeptide (VIP) immunoreactive nerves. *Life Sci* **22**:773-780

Leah JD, Cameron AA, Kelly WL, Snow PJ (1985) Coexistence of peptide immunoreactivity in sensory neurons of the cat. *Neuroscience* **16**:683-690

Lee Y, Kawai Y, Shiosaka S, Takami K, Kiyama H, Hillyard CJ, Girgis S, MacIntyre I, Emson PC, Tohyama M (1985a) Coexistence of calcitonin gene-related peptide and substance P-like peptide in single cells of the trigeminal ganglion of the rat: immunohistochemical analysis. *Brain Res* **330**:194-196

Lee Y, Takami K, Kawai Y, Girgis S, Hillyard CJ, MacIntyre I, Emson PC (1985b) Distribution of calcitonin gene-related peptide in the rat peripheral nervous system with reference to its coexistence with substance P. *Neuroscience* **15**:1227-1237

Le Grevès P, Nyberg F, Hökfelt T, Terenius L (1989) Calcitonin gene-related peptide is metabolized by an endopeptidase hydrolyzing substance P. *Regul Pept* **25**:277-286

Le Grevès P, Nyberg F, Terenius L, Hökfelt T (1985) Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. *Eur J Pharmacol* **115**:309-311

Lembeck F, Holzer P, Schweditsch M, Gamse R (1978) Elimination of substance P from the circulation of the rat and its inhibition by bacitracin. *Naunyn Schmiedebergs Arch Pharmacol* **305**:9-16

Levine M (1928) Oxyphile cells in the parathyroid glands of the cow and steer. *Anat Rec* **39**:293-299

Lundberg JM, Hökfelt T, Hemsén A, Theodorsson-Norheim E, Pernow J, Hamberger B, Golstein M (1986) Neuropeptide Y-like immunoreactivity in adrenaline cells of adrenal medulla and in tumors and plasma of pheochromocytoma patients. *Regul Pept* **13**:169-182

Lundberg JM, Franco-Cereceda A, Hua X, Hökfelt T, Fischer JA (1985) Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. *Eur J Pharmacol* **108**:315-319

MacGregor RR, Chu LLH, Hamilton JW, Cohn DV (1973) Studies on the subcellular localization of proparathyroid hormone and parathyroid hormone in the bovine parathyroid gland: separation of newly synthesized from mature forms. *Endocrinology* **93**:1387-1397

MacGregor RR, Sarras Jr. MP, Houle A, Cohn DV (1983) Primary monolayer cell culture of bovine parathyroids: effects of calcium, isoproterenol and growth factors. *Mol Cell Endocrinol* **30**:313-328

Maggi CA, Meli A (1988) The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen Pharmacol* **19**:1-43

Marasco WA, Showell HJ, Becker EL (1981) Substance P binds to the formylpeptide chemotaxis receptor on the rabbit neutrophil. *Biochem Biophys Res Commun* **99**:1065-1072

Marshall RW (1976) Plasma fractions. In: Nordin BEC (ed) Calcium, phosphate and magnesium metabolism. Churchill Livingstone, London, pp 162-175

Mayer GP, Hurst JG (1978) Sigmoidal relationship between parathyroid hormone secretion rate and plasma calcium concentration in calves. *Endocrinology* **102**:1036-1042

Mazzocchi G, Meneghelli V, Serafini MT (1967) The fine structure of the parathyroid glands in the normal, the rachitic and the bilaterally nephrectomized rat with special interest to their secretory cycle. *Acta Anat (Basel)* **68**:550-566

McEwan J, Larkin S, Davies G, Chierchia S, Brown M, Stevenson J, MacIntyre I, Maseri A (1986) Calcitonin gene-related peptide: a potent dilator of human epicardial coronary arteries. *Circulation* **74**:1243-1247

McSheehy PMJ, Chambers TJ (1986) Osteoblast-like cells in the presence of parathyroid hormone release soluble factor that stimulates osteoclastic bone resorption. *Endocrinology* **119**:1654-1659

Metz SA, Deftos LJ, Baylink DJ, Robertson RP (1978) Neuroendocrine modulation of calcitonin and parathyroid hormone in man. *J Clin Endocrinol Metab* **47**:151-159

Michelangeli VP, Findlay DM, Fletcher A, Martin TJ (1986) Calcitonin gene-related peptide (CGRP) acts independently of calcitonin on cyclic AMP formation in clonal osteogenic sarcoma cells (UMR 106-01). *Calcif Tissue Int* **39**:44-48

Michelangeli VP, Fletcher AE, Allan EH, Nicholson GC, Martin TJ (1989) Effects of calcitonin gene-related peptide on cyclic AMP formation in chicken, rat, and mouse bone cells. *J Bone Miner Res* **4**:269-272

Mikhail Y (1971) Intrinsic nerve supply of the thyroid and parathyroid glands. *Acta Anat (Basel)* **80**:152-159

Minami Y, Onoue H, Toda N (1989) Extraluminally applied acetylcholine and substance P on the release of EDRF. *Jpn J Pharmacol* **50**:362-365

Mitsuhashi M, Payan DG (1987) The mitogenic effects of vasoactive neuropeptides on cultured smooth muscle cell lines. *Life Sci* **40**:853-861

Miyaura C, Tamura T, Owan I, Akatsu T, Suda T (1992) The mechanism of action of amylin in osteoclasts and osteoblasts. *Bone Miner* **17 (Suppl 1)**:111 (Abst. 148)

Moore KL (1988) The developing human, 4th Ed. W.B. Saunders Co., Philadelphia, p 176

Morel G, Besson J, Rosselin G, Dubois PM (1982) Ultrastructural evidence for endogenous vasoactive intestinal peptide-like immunoreactivity in the pituitary gland. *Neuroendocrinology* **34**:85-89

Morii H, Fujita T, Okinaka S (1963) Effect of vagotomy and atropine on recovery from induced hypocalcemia. *Endocrinology* **72**:173-179

Morris HR, Panico M, Etienne T, Tippins J, Girgis SI, MacIntyre I (1984) Isolation and characterization of human calcitonin gene-related peptide. *Nature* **308**:746-748

Morrissey JJ, Cohn DV (1978) The effects of calcium and magnesium on the secretion of parathormone and parathyroid secretory protein by isolated porcine parathyroid cells. *Endocrinology* **103**:2081-2090

Morrissey JJ, Cohn DV (1979) Secretion and degradation of parathormone as a function of intracellular maturation of hormone pools: modulation by calcium and dibutyryl cyclic AMP. *J Cell Biol* **83**:521-528

Mortimer ST, Hanley DA, Stell WK (1990) Immunohistochemical identification of calcitonin gene-relate peptide and substance P in nerves of the bovine parathyroid gland. *Cell Tissue Res* **261**:339-345

Mulderry PK, Ghatei MA, Rodrigo J, Allen JM, Rosenfeld MG, Polak JM, Bloom SR (1985) Calcitonin gene-related peptide in cardiovascular tissues of the rat. *Neuroscience* **14**:947-954

Munger BL, Roth SI (1963) The cytology of the normal parathyroid glands of man and Virginia deer. *J Cell Biol* **16**:379-400

Nawa H, Hirose T, Takashima H, Inayama S, Nakanishi S (1983) Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precursor. *Nature* **306**:32-36

New HV, Mudge AW (1986) Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature* **323**:809-811

Nicholson GC, Moseley JM, Sexton PM, Mendelsohn FAO, Martin TJ (1986) Abundant calcitonin receptors in isolated rat osteoclasts. Biochemical and autoradiographic characterization. *J Clin Invest* **78**:355-360

Nilsson J, von Euler AM, Dalsgaard C-J (1985) Stimulation of connective tissue cell growth by substance P and substance K. *Nature* **315**:61-63

Norberg K-A, Persson B, Granberg P-O (1975) Adrenergic innervation of the human parathyroid glands. *Acta Chir Scand* **141**:319-322

Nyberg F, Le Grevès P, Sundqvist C, Terenius L (1984) Characterization of substance P(1-7) and (1-8) generating enzyme in human cerebrospinal fluid. *Biochem Biophys Res Commun* **125**:244-250

Ojeda SR, Griffin JE (1988) Organization of the endocrine system. In: Griffin JE, Ojeda SR (eds) Textbook of endocrine physiology. Oxford University Press, New York, p 3

Oku R, Satoh M, Fujii N, Otaka A, Yajima H, Takagi H (1987) Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. *Brain Res* **403**:350-354

Parthemore JG, Deftos LJ (1978) Calcitonin secretion in normal human subjects. *J Clin Endocrinol Metab* **47**:184-188

Parthemore JG, Deftos LJ, Bronzert D (1975) The regulation of calcitonin in normal human plasma as assessed by immunoprecipitation and immunoextraction. *J Clin Invest* **56**:835-841

Parthemore JG, Roos BA, Parker DC, Kripke DF, Avioli LV, Deftos LJ (1978) Assessment of acute and chronic changes in parathyroid hormone secretion by a radioimmunoassay with predominant specificity for the carboxy-terminal region of the molecule. *J Clin Endocrinol Metab* **47**:284-289

Payan DG (1985) Receptor-mediated mitogenic effects of substance P on cultured smooth muscle cells. *Biochem Biophys Res Commun* **130**:104-109

Payan DG, Brewster DR, Goetzl EJ (1983) Specific stimulation of human T lymphocytes by substance P. *J Immunol* **131**:1613-1615

Payan DG, Levine JD, Goetzl EJ (1984) Modulation of immunity and hypersensitivity by sensory neuropeptides. *J Immunol* **132**:1601-1604

Pearce AGE, Polak JM (1971) Cytochemical evidence for the neural crest origin of mammalian ultimobranchial C cells. *Histochemie* **27**:96-102

Peck R (1987) Neuropeptides modulating macrophage function. *Ann N Y Acad Sci* **496**:264-270

Pedersen KO (1972) Protein-bound calcium in human serum. Quantitative examination of binding and its variables by a molecular binding model and clinical chemical implications for measurement of ionized calcium. *Scand J Clin Lab Invest* **30**:321-329

Pettersson M, Ahrén B, Bottcher G, Sundler F (1986) Calcitonin gene-related peptide: occurrence in pancreatic islets in the mouse and the rat and inhibition of insulin secretion in the mouse. *Endocrinology* **119**:865-869

Pick J (1970) The principles of the autonomic nervous system. In: The autonomic nervous system. Lippincott JB, Toronto, p 23

Prior M, Green F, Lopez A, Balu A, De Sanctis GT, Fick G (1990) Capsaicin pretreatment modifies hydrogen sulphide-induced pulmonary injury in rats. *Toxicol Pathol* **18**:279-288

Przepiorka D, Baylin SB, McBride OW, Testa JR, de Bustros A, Nelkin BD (1984) The human calcitonin gene is located on the short arm of chromosome 11. *Biochem Biophys Res Commun* **120**:493-499

Rawlins TGR, Yrjönen T (1978) Calculation of RIA results using the spline function. *International Laboratory* **Nov/Dec**

Raybuck HE (1952) The innervation of the parathyroid glands. *Anat Rec* **112**:117-123

Regoli D, Drapeau G, Dion S, Couture R (1988) New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. *Trends Pharmacol Sci* **9**:290-295

Reimann I, Christensen SB (1977) A histological demonstration of nerves in subchondral bone. *Acta Orthop Scand* **48**:345-352

Reinecke M, Weihe E, Forssmann WG (1980) Substance P-immunoreactive nerve fibers in the heart. *Neurosci Lett* **20**:265-269

Rhinehart DA (1912) The nerves of the thyroid and parathyroid bodies. *Am J Anat* **13**:91-102

Romeo HE, Solveyra CG, Vacas MI, Rosenstein RE, Barontini M, Cardinali DP (1986) Origins of the sympathetic projections to rat thyroid and parathyroid glands. *J Auton Nerv Syst* **17**:63-70

Roos BA, Deftos L (1979) Parathyroid hormone. In: Jaffe BM, Behrman HR (eds) Methods of hormone radioimmunoassay, 2nd Ed. Academic Press, Inc., London pp 403-406

Roos BA, Fischer JA, Pignat W, Alander CB, Raisz LG (1986) Evaluation of the *in vivo* and *in vitro* calcium-regulating actions of noncalcitonin peptides produced via calcitonin gene expression. *Endocrinology* **118**:46-51

Rosenfeld MG, Amara SG, Roos BA, Ong EG, Evans RM (1981) Alternate expression of the calcitonin gene associated with RNA polymorphism. *Nature* **290**:63-65

Rosenfeld MG, Mermod J-J, Amara SG, Swanson LW, Sawchenko PE, Rivier J, Vale WW, Evans RM (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* **304**:129-135

Roth SI, Munger BL (1962) The cytology of the adenomatous, atrophic, and hyperplastic parathyroid glands of man. A light- and electron-microscopic study. *Virchows Arch [A]* **335**:389-410

Roth SI, Schiller AL (1976) Comparative anatomy of the parathyroid glands. In: Greep RO, Astwood EB (eds) Handbook of Physiology, Section 7: Endocrinology (Vol VII. Parathyroid Gland). American Physiological Society, Washington, p 293

Rotsztejn WH, Benoist L, Besson J, Beraud G, Bluet-Pajot MT, Kordon C, Rosselin G, Duval J (1980) Effect of vasoactive intestinal peptide (VIP) on the release of adenohypophyseal hormones from purified cells obtained by unit gravity sedimentation. *Neuroendocrinology* **31**:282-286

Ruff MR, Wahl SM, Pert CB (1985) Substance P receptor-mediated chemotaxis of human monocytes. *Peptides* **6(Suppl 2)**:107-111

Schultz GS, Sarras Jr. MP, Gunther GR, Hull BE, Alicea HA, Gorelick FS, Jamieson JD (1980) Guinea pig pancreatic acini prepared with purified collagenase. *Exp Cell Res* **130**:49-62

Shepherd GM (1988) Neurobiology, 2nd Ed. Oxford University Press, Inc., New York, pp 254-255

Shih C, Wang TM (1992) Intravenous administration of neuroactive calcitonin gene-related peptide enhances osteogenesis. *Bone Miner* **17 (Suppl 1)**:200 (Abst. 497)

Shoumura S, Iwasaki Y, Ishizaki N, Emura S, Hayashi K, Yamahira T, Shoumura K, Isono H (1983) Origin of autonomic nerve fibers innervating the parathyroid gland in the rabbit. *Acta Anat (Basel)* **115**:289-295

Siegel S, Castellan Jr. NJ (1988) Nonparametric statistics for the behavioral sciences, 2nd Ed. McGraw-Hill Book Co., New York, pp 128-132

Sigrist S, Francol-Cereceda A, Muff R, Henke H, Lundberg JM, Fischer JA (1986) Specific receptor and cardiovascular effects of calcitonin gene-related peptide. *Endocrinology* **119**:381-389

Sinha TK, Miller S, Fleming J, Khairi R, Edmondson J, Johnston Jr. CC, Bell NH (1975) Demonstration of a diurnal variation in serum parathyroid hormone in primary and secondary hyperparathyroidism. *J Clin Endocrinol Metab* **41**:1009-1013

Skofitsch G, Jacobowitz DM (1985) Calcitonin gene-related peptide coexists with substance P in capsaicin sensitive neurons and sensory ganglia of the rat. *Peptides* **6**:747-754

Stanisz AM, Befus D, Bienenstock J (1986) Differential effects of vasoactive intestinal peptide, substance P, and somatostatin on immunoglobulin synthesis and proliferations by lymphocytes from Peyer's patches, mesenteric lymph nodes, and spleen. *J Immunol* **136**:152-156

Stead RH, Tomioka M, Quinonez G, Simon GT, Felten SY, Bienenstock J (1987) Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. *Proc Natl Acad Sci USA* **84**:2975-2979

Steenbergh PH, Höppener JWM, Zandberg J, Lips CJM, Jansz HS (1985) A second human calcitonin/CGRP gene. *FEBS Lett* **183**:403-407

Sternini C, Brecha N (1986) Immunocytochemical identification of islet cells and nerve fibers containing calcitonin gene-related peptide-like immunoreactivity in the rat pancreas. *Gastroenterology* **90**:1155-1163

Stewart AF, Broadus AE (1987) Mineral Metabolism. In: Felig P, Baxter JD, Broadus AE, Frohman LA (eds) Endocrinology and Metabolism, 2nd Ed. McGraw-Hill, Inc., Toronto, p 1317

Struthers AD, Brown MJ, Beacham JL, Morris HR, MacIntyre I, Stevenson JC (1985) The acute effect of human calcitonin gene-related peptide in man. *J Endocrinol* **104 (Suppl)**:129 (Abst. 225)

Sundler F, Alumets J, Brodin E, Dahlberg K, Nilsson G (1977) Perivascular substance P-immunoreactive nerves in tracheobronchial tissue. In: von Euler US, Pernow B (eds) Substance P. Raven Press, New York, pp 271-273

Takami K, Kawai Y, Shiosaka S, Lee Y, Girgis S, Hillyard CJ, MacIntyre I, Emson PC, Tohyama M (1985) Immunohistochemical evidence for the coexistence of calcitonin gene-related peptide- and choline acetyltransferase-like immunoreactivity in neurons of the rat hypoglossal, facial and ambiguus nuclei. *Brain Res* **328**:386-389

Targovnik JH, Rodman JS, Sherwood LM (1971) Regulation of parathyroid hormone secretion *in vitro*: quantitative aspects of calcium and magnesium ion control. *Endocrinology* **88**:1477-1482

Tatemoto K, Lundberg JM, Jörnvall H, Mutt V (1985) Neuropeptide K: isolation, structure and biological activities of a novel brain tachykinin. *Biochem Biophys Res Commun* **128**:947-953

Taylor PE, Byers MR, Redd PE (1988) Sprouting of CGRP nerve fibres in response to dentin injury in rat molars. *Brain Res* **461**:371-376

Tippins JR, Morris HR, Panico M, Etienne T, Bevis P, Girgis S, MacIntyre I, Azria M, Attinger M (1984) The myotropic and plasm-calcium modulating effects of calcitonin-gene related peptide (CGRP). *Neuropeptides* **4**:425-434

Tremblay G, Carter GE (1961) Histochemical study of oxidative enzymes in the human parathyroid. *Endocrinology* **69**:658-661

Tuchscherer MM, Seybold VS (1985) Immunohistochemical studies of substance P, cholecystokinin-octapeptide and somatostatin in dorsal root ganglia of the rat. *Neuroscience* **14**:593-605

Uddman R, Edvinsson L, Ekblad E, Håkanson R, Sundler F (1986) Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. *Regul Pept* **15**:1-23

Unsicker K (1971) On the innervation of mammalian endocrine glands (anterior pituitary and parathyroids). *Z Zellforsch* **121**:283-291

Varndell IM, Polak JM, Allen JM, Terenghi G, Bloom SR (1984) Neuropeptide tyrosine (NPY) immunoreactivity in norepinephrine-containing cells and nerves of the mammalian adrenal gland. *Endocrinology* **114**:1460-1462

Vijayan E, McCann SM (1979) *In vivo* and *in vitro* effects of substance P and neurotensin on gonadotropin and prolactin release. *Endocrinology* **105**:64-68

von Euler US, Gaddum JH (1931) An unidentified depressor substance in certain tissue extracts. *J Physiol (Lond)* **72**:74-87

Vora NM, Bowser EN, Hargis GK, Johnson PA, Kukreja SC, Williams GA (1980) Effect of cervical sympathetic stimulation on parathyroid hormone secretion in the rat. *Clin Res* **28**:409A (Abst.)

Vora NM, Kukreja SC, Bowser EN, Johnson PA, Hargis GK, Williams GA (1978) Role of endogenous adrenergic tone in parathyroid hormone and calcitonin secretion. *Clin Res* **26**:685A (Abst.)

Wanaka A, Matsuyama T, Yoneda S, Kamada T, Emson PC, Hillard J, Girgis SJ, MacIntyre I, Tohyama M (1987) Distribution patterns of calcitonin gene-related peptide-containing fibers in the wall of the three different arteries: an immunohistochemical study. *Cell Mol Biol* **33**:201-209

Wanaka A, Matsuyama T, Yoneda S, Kimura K, Kamada T, Girgis S, MacIntyre I, Emson PC, Tohyama M (1986) Origins and distribution of calcitonin gene-related peptide-containing nerves in the wall of the cerebral arteries of the guinea pig with special reference to the coexistence with substance P. *Brain Res* **369**:185-192

Wener JA, Gorton SJ, Raisz LG (1972) Escape from inhibition of resorption in cultures of fetal bone treated with calcitonin and parathyroid hormone. *Endocrinology* **90**:752-759

Wharton J, Polak JM, Probert L, De Mey J, McGregor GP, Bryant MG, Bloom SR, (1981) Peptide containing nerves in the ureter of the guinea-pig and cat. *Neuroscience* **6**:969-982

Wideman Jr. RF (1980) Innervation of the parathyroid in the European starling (*Sturnus vulgaris*). *J Morphol* **166**:65-80

Wiesenfeld-Hallin Z, Hökfelt T, Lundberg JM, Forssmann WG, Reinecke M, Tschopp FA, Fischer JA (1984) Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. *Neurosci Lett* **52**:199-204

Williams GA, Kukreja SC, Longley RS, Bowser EN, Hargis GK, Vora NM, Henderson WJ (1985) Effect of the parasympathetic system on secretion of parathyroid hormone. *Metabolism* **34**:612-615

Williams GA, Longley RS, Bowser EN, Hargis GK, Kukreja SC, Vora NM, Johnson PA, Jackson BL, Kawahara WJ, Henderson WJ (1981) Parathyroid hormone secretion in normal man and in primary hyperparathyroidism: role of histamine H_2 receptors. *J Clin Endocrinol Metab* **52**:122-127

Yamamoto I, Kitamura N, Aoki J, Shigeno C, Hino M, Asonuma K, Torizuka K, Fujii N, Otaka A, Yajima H (1986) Human calcitonin gene-related peptide possesses weak inhibitory potency of bone resorption *in vitro*. *Calcif Tissue Int* **38**:339-341

Yeghiayan E, Rojo-Ortega JM, Genest J (1972) Parathyroid vessel innervation: an ultrastructural study. *J Anat* **112**:137-142

Zabel M, Biela-Jacek I, Surdyk J, Dietel M (1987) Studies on localization of calcitonin gene-related peptide (CGRP) in the thyroid-parathyroid complex. *Virchows Arch [A]* **411**:569-573

Zaidi M, Bevis PJR, Abeyasekera G, Girgis SI, Wimalawansa SJ, Morris HR, MacIntyre I (1986) The origin of circulating calcitonin gene-related peptide in the rat. *J Endocrinol* **110**:185-190

Zaidi M, Bevis PJR, Girgis SI, Lynch C, Stevenson JC, MacIntyre I (1985) Circulating CGRP comes from the perivascular nerves. *Eur J Pharmacol* **117**:283-284

Zaidi M, Chambers TJ, Bevis PJR, Beacham JL, Gaines Das RE, MacIntyre I (1988) Effects of peptides from the calcitonin genes on bone and bone cells. *Q J Exp Physiol* **73**:471-485

Zaidi M, Chambers TJ, Gaines Das RE, Morris HR, MacIntyre I (1987a) A direct action of human calcitonin gene-related peptide on isolated osteoclasts. *J Endocrinol* **115**:511-518

Zaidi M, Fuller K, Bevis PJR, Gaines Das RE, Chambers TJ, MacIntyre I (1987b) Calcitonin gene-related peptide inhibits osteoclastic bone resorption: a comparative study. *Cell Tissue Res* **40**:149-154

Zamboni L, De Martino C (1967) Buffered picric acid-formaldehyde: a new, rapid fixative for electron microscopy. *J Cell Biol* **35**:148A (Abst. 307)

Zawadzki JV, Furchgott RF, Cherry P (1981) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by substance P. *Fed Proc* **40**:689 (Abst. 2627)

Zawistowski S (1966) Histochemical studies on the adrenergic innervation of the parathyroid gland of albino rat. *Z Mikrosk Anat Forsch* **74**:39-45