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Protein Kinase C Isoenzymes in the Bovine Parathyroid and their Involvement in the Degradation and Secretion of Parathyroid Hormone

by

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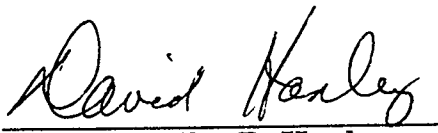
The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Protein Kinase C Isoenzymes in the Bovine Parathyroid and their Involvement in the Degradation and Secretion of Parathyroid Hormone" submitted by Farzana Shivji in partial fulfillment of the requirements for the degree of Master of Science.



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Abstract

Western blot analysis was used to screen bovine parathyroid cell fractions for protein kinase C (PKC) α, β, δ, ε, η, θ, and ζ isoenzymes. PKCs α, β, ε, and ζ were identified by this method. Additionally, a protein representing PKC ζ' or PKC λ was identified. Hydroxylapatite chromatography confirmed the presence of the classical PKCs (cPKCs) α and β, but failed to detect cPKC γ.

Membrane association of cPKCs and parathyroid hormone (PTH) secretion of parathyroid tissue were examined in response to high and low calcium treatments, in the presence and absence of phorbol myristate acetate (PMA). Only the low calcium treatment resulted in membrane association of PKC α. Both PKC β membrane association and PTH secretion were greatest in the low calcium treatment, followed by the high calcium plus PMA treatment.

Purified PKC phosphorylated bovine and human PTH. Phosphopeptide analysis revealed that serine-48 is phosphorylated by PKC.

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Thank you for pointing out the roses, as well as the thorns,

and for letting me experience them for myself.

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List of Abbreviations

AA	arachidonic acid
aPKC	atypical protein kinase C
ATP	adenosine triphosphate
bPTH	bovine parathyroid hormone
Ca	ionized calcium
Ca_e	extracellular calcium
Ca_i	intracellular calcium
cAMP	3'-5' cyclic adenosine monophosphate
cPKC	classical protein kinase C
cpm	counts per minute
DAG	diacylglycerol
EDTA	ethylenediamine-tetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
GTP	guanosine triphosphate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hPTH	human parathyroid hormone
IP_3	inositol 1,4,5 trisphosphate
IP_4	inositol 1,3,4,5 tetrakisphosphate
M	molar
MBP	myelin basic protein
mM	millimolar
nPKC	novel protein kinase C
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonyl fluoride
PTH	parathyroid hormone
RIA	radioimmunoassay
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
uCi	microcurie
uM	micromolar

Introduction

A recurrent theme found in the study of seemingly diverse biological systems is the repeated use of common molecular phenomena. This principle is evident in the study of signal transduction pathways. These are the processes by which a given cell responds to extracellular signals which culminate in diverse manifestations such as secretion, gene expression and cellular differentiation. Indeed, if each extracellular stimulus required the use of a specialized intracellular pathway, the amount of genetic information that an organism would be required to store would be prohibitive. Through a variety of signal transduction pathways a given cell is able to respond to a variety of stimuli. Due to similarities in the signaling molecules used, the cell is endowed with the ability to effectively respond to many aspects of its environment in a coordinated manner. In many cells, calcium is one such common molecule. Increases in intracellular calcium levels are used in a variety of signal transduction pathways to increase secretion. These pathways frequently utilize reversible protein phosphorylation which is mediated by kinases and phosphatases that are directly or indirectly dependent on calcium. It is noteworthy, however, that in the parathyroid, secretion is enhanced by a decrease in intracellular calcium. This cell type has been shown to contain a variety of signaling molecules found in other secretory cells, and thus probably represents a variation in the common theme of secretion processes. It is the goal of this thesis to identify whether the variations in the parathyroid secretory response may be due to specialization of individual members of the protein kinase C (PKC) family. In doing so, it is hoped that a greater

understanding of the specialization of protein kinase C isoenzymes as well as secretory responses will result.

1.1 Protein Kinase C

1.1.1 Discovery and structure

Protein Kinase C (PKC) was originally isolated from rat brain in 1977 by Inoue *et al.* This group described PKC as the precursor to a calcium- and cyclic nucleotide- independent protein kinase they termed protein kinase M (PKM) (Inoue *et al*, 1977, Takai *et al*, 1977). In later studies it was found that this precursor actually exhibited kinase activity in the presence of calcium and phospholipids (Nishizuka *et al*, 1979). Further, it was found that this kinase activity co-purified with phorbol ester binding activity (Ashendel *et al*, 1983). Phorbol esters, such as phorbol myristate acetate (PMA) (also known as tetradecanoyl phorbol acetate (TPA)), are currently thought to mimic the action of diacylglycerol (DAG), another activator of this kinase (Thomas *et al*, 1987). Using classical protein purification techniques, three Ca- and phospholipid-dependent, phorbol ester-binding protein kinases were identified in a large variety of species and tissues. Genetic and structural analysis indicated a great degree of homology between these kinases and they were collectively termed the protein kinase C family. The use of cDNA probes at low stringency has revealed at least ten isoenzymes of protein kinase C. Although these isoenzymes share a large degree of sequence homology, they exhibit diversity in their structural and activational characteristics. The ten PKC isoenzymes have been subdivided into three subgroups based on their structural characteristics and cofactor requirements (Nishizuka, 1992). These three groups are: group A, the

classical PKCs (cPKCs); group B, the novel PKCs (nPKCs); and group C, the atypical PKCs (aPKCs).

Figure 1.1 illustrates the structural differences between the three groups of PKCs (reviewed by Nishizuka, 1992, Hug and Sarre, 1993, and Dekker and Parker, 1994). Four conserved (C1-C4) and five variable (V1-V5) regions have been identified. All known PKCs contain an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain. The catalytic domain contains a highly conserved ATP binding site, and displays cofactor-independent kinase (PKM) activity when proteolytically cleaved from the regulatory domain. A pseudosubstrate sequence, containing a PKC substrate consensus sequence with an alanine in place of the substrate serine or threonine, is found in the regulatory domain of all identified PKCs. This sequence is thought to interact with the catalytic site of PKC in the absence of activator molecules. The differences in the specific pseudosubstrate sequences between individual isoenzymes is thought to indicate the differences in their substrate specificities. Regions which are thought to influence the activational characteristics of the PKCs are found in the regulatory domain and are the basis for the classification of the individual isoenzymes into the three groups.

1.1.2 The classical group of PKC isoenzymes

The three classical PKC isoenzymes were the first PKCs discovered, and are actually the only group of PKCs that require calcium for their activation. Additionally, the members of this group are activated by acidic phospholipids, various cis-unsaturated fatty acids, and DAG / phorbol ester (Kikkawa *et al*, 1983). Included in this group are PKC α, PKC β (PKC

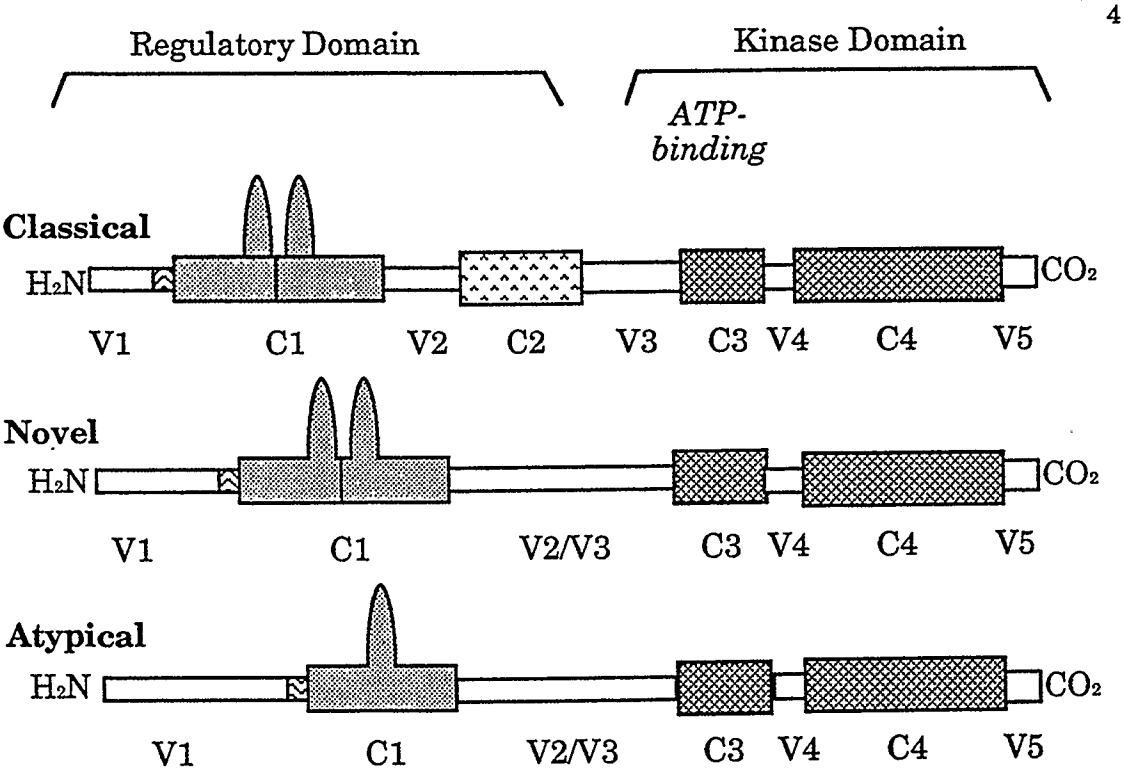

Figure 1.1 Schematic representation of the domain structure of classical, novel and atypical PKC isoenzymes. The four conserved regions (C1-C4) and the five variable regions (V1-V5) are shown. The C1 region is divided into two parts, each corresponding to a cysteine-rich zinc finger-like sequence. This region is responsible for high-affinity binding to DAG/phorbol esters. The atypical PKCs either do not posses both regions of the C1 domain (aPKCs ζ and λ, as shown), or contain both zinc finger motifs but are separated by a greater number of residues (aPKC μ, not represented in the schematic), resulting in their inability to bind phorbol esters. The C2 domain confers the ability to bind calcium ions with high affinity, and is found only in the classical PKCs (cPKCs α, β, and γ).

βI, PKC βII) and PKC γ. These isoenzymes have been identified in a wide variety of mammalian species (Dekker and Parker, 1994). PKC γ is a brain specific isoenzyme (Ohno *et al*, 1988), whereas PKC α is expressed in a wide variety of tissues. The two splice variants of PKC β, PKCs βI and βII, (Coussens *et al*, 1987), appear to be more selective in their tissue distribution. Hydroxylapatite chromatography is frequently used to resolve PKCs α, β and γ (Huang *et al*, 1986). Studies using individual hydroxylapatite-purified cPKC isoenzymes indicate that these differ in their calcium-, phospholipid- and DAG (or phorbol ester)- sensitivity as well as their preference for specific free fatty acid activators, substrates, and susceptibility to proteolytic conversion into PKM (reviewed by Kikkawa *et al*, 1987, Huang *et al*, 1988).

1.1.3 The novel group of PKC isoenzymes

Unlike the cPKC isoenzymes, the novel PKCs (nPKCs) do not display any calcium requirement for their activation. This calcium independence of this group of PKC isoenzymes is thought to be due to the absence of C-2, a region which is present in the amino-terminal regulatory domain of the cPKCs (Osada *et al*, 1990) (see Figure 1.1). Like the cPKCs, the nPKC isoenzymes are activated by acidic phospholipids, various cis-unsaturated fatty acids and DAG / phorbol ester (Newton, 1993). This group includes the δ, ε, η, and θ PKC isoenzymes. Although these specific isoenzymes are found in all species examined, they display a large degree of tissue specificity. PKC δ is found in a variety of tissues (Maizels *et al*, 1990, Cutler *et al*, 1993, Leibersperger *et al*, 1991, Ogita, *et al*, 1992, Oudinet, *et al*, 1992). Several reports indicate that this isoenzyme is primarily found in the particulate fraction of the cells, which primarily contains cellular

membranes. The observed membrane-association of PKC δ is proposed to indicate specialization of this PKC isoenzyme (Leibersperger *et al*, 1991, Ogita, *et al*, 1992). PKC ε shows a greater tissue selectivity than PKC δ and is usually found in the cytosolic, or soluble, fractions of resting cells (Schaap and Parker, 1990, Koide, *et al*, 1992, Saido *et al*, 1992). PKCs η and θ display a great deal of tissue specificity. PKC η shows greatest sequence similarity to PKC ε, and is expressed primarily in epithelial tissue (Bacher *et al*, 1991, Osada *et al*, 1990). PKC θ is expressed predominantly in skeletal muscle and shows greatest sequence similarity to PKC δ (Osada *et al*, 1992). In addition to differences in localization and tissue distribution, the novel PKCs differ from one another in their substrate specificity and activational requirements. Some reports indicate that, depending on the substrate used, these isoenzymes differ from the cPKCs in their inhibition by classical PKC inhibitors such as H-7 and K252b (Oudinet *et al*, 1992, Koide *et al*, 1992), and their unresponsiveness to calcium.

1.1.4 The atypical group of PKC isoenzymes

Three members of the atypical PKC group have been identified. These include PKC ζ, PKC λ (which is also called PKC ι (Selbie *et al*, 1993)), and PKC μ. Like the nPKCs, the atypical PKCs differ from the cPKCs in that they do not require calcium for their activation. In contrast to the other two groups of PKC isoenzymes, the aPKCs are not activated by phorbol esters *in vitro* (Nishizuka, 1992). This difference in phorbol ester-responsiveness is thought to be due to a change in the C1 region of the amino-terminal regulatory domain (see Figure 1.1). Unlike the cPKCs and nPKCs which have two cysteine-rich zinc fingers within 15 amino acids of each other in the C1 region, the aPKCs either have only one such motif (as in PKC ζ and

λ) or two cysteine-rich regions but with a greater separation between them (as in PKC μ) (Johannes *et al*, 1994). All three identified atypical PKCs show a wide tissue distribution. PKC ζ and λ share a large degree of sequence similarity, and are activated *in vitro* by phospholipids and unsaturated fatty acids, especially arachidonic acid (Nakanashi and Exton, 1992, Akimoto *et al*, 1994). Although *in vitro* studies indicate these isoenzymes are not activated by phorbol esters, it is possible that *in vivo* they are secondarily affected by these compounds (Ways *et al*, 1992, Zhou *et al*, 1994). PKC μ has been recently identified, and little is known about this isoenzyme. Initial findings indicate that this isoenzyme is different from the other PKC isoenzymes in that it contains a potential signal peptide in its amino-terminal region as well as a potential transmembrane domain. These findings, in conjunction with the large tissue expression of this isoenzyme indicate a potential role for PKC μ in the early stages of signal transduction processes (Johannes *et al*, 1994).

1.1.5 Specialization of PKC isoenzymes

It is evident that a large number of isoenzymes are found in all species examined (Dekker and Parker, 1994). Furthermore, the sequence variations between individual isoenzymes is much greater than the variation between species for a particular isoenzyme. These observations suggest that a variety of PKC isoenzymes are necessary for biological functions. The finding that individual isoenzymes display different activational requirements as well as tissue distribution patterns, suggest that individual PKC isoenzymes perform specialized tasks within a given cell (reviewed by Nishizuka, 1992, Hug and Sarre, 1993, Dekker and Parker, 1994). A wide variety of cellular functions have been attributed to PKC

activation. These include gene expression, cell proliferation, smooth muscle contraction, modulation of ion conductance, and secretion (Kikkawa *et al*, 1989 and references therein).

1.1.6 Possible mechanisms of cellular regulation by PKC

It is clear that PKC plays a regulatory role in many cell functions. Although little is known about the exact mechanisms by which PKC regulates cellular activity, a few common themes have been proposed. PKC phosphorylates serine and/or threonine residues in substrate proteins. This phosphorylation may a) alter the catalytic activity of enzymatic substrates, b) alter the conductivity or sensitivity of ion channels or receptors, c) alter the binding of substrate proteins to other proteins or cellular structures. PKC activity has been directly attributed to all of these phenomena in a variety of cell types (Nishizuka, 1989, Hug and Sarre, 1993, Nishizuka, 1992, Dekker and Parker 1994). A multitude of other consequences of phosphorylation by PKC remain to be explored. One possibility is that phosphorylation by PKC may alter the susceptibility of the substrate protein to enzymatic modification. The role of PKC in the regulation of proteolytic cleavage of substrate proteins has been suggested (Rogers *et al*, 1986, Wang *et al*, 1989, Friedrich and Aszodi, 1991). Recently, phosphorylation of one protein, connexin-32, by PKC has been demonstrated to alter its susceptibility to proteolytic cleavage (Elvira *et al*, 1992, Elvira *et al*, 1994). It remains to be determined whether this is a general mechanism by which PKC regulates cellular activities.

1.1.7 Regulation of PKC activity

The large variety of activities which have been attributed to PKC activation suggests that PKC activity must be stringently controlled in order

for efficient cell function. In the analysis of PKC function and regulation, it is important to examine which isoenzymes are present within a given cell. Due to the diverse activational requirements for each isoenzyme, it is evident that the cellular distribution and production of these activating molecules determines the level of activity of each isoenzyme. It is important to note that PKC activation has been implicated in the regulation of the cellular levels of a wide range of PKC activators. Activation of PKC appears to result in both negative feedback as well as feedforward regulation of its own activity (Nishizuka, 1992). Taken together with isoenzyme specialization, it is conceivable that activation of one isoenzyme, or group of isoenzymes, results in the regulation of other isoenzymes.

Another factor determining PKC activity is its localization within the cell. It is clear that, in order for activation to occur, PKC must have access to its activators. For those isoenzymes known to be activated by phospholipids, proximity to phospholipid-rich compartments is a key factor in their activation within the cell. For the cPKC and nPKCs, one means of identifying activation within the cell has been the measurement of membrane association (Girard *et al*, 1985, Thomas *et al*, 1987, Fournier *et al*, 1989). Another factor in the regulation of PKC activity is probably the substrate specificity of the isoenzyme(s) involved, and the localization of these substrates in relation to the isoenzyme(s) (Hug and Sarre, 1993, Nishizuka, 1992, Dekker and Parker, 1994).

Proteolytic degradation of PKC also plays a role in the regulation of PKC activity. *In vitro*, PKC can be converted into a cofactor-independent kinase (PKM) by a variety of proteases (Inoue *et al*, 1977). This conversion has been demonstrated for both the cPKC and nPKC isoenzymes (reviewed

by Kikkawa *et al*, 1989). In the cell, it is thought that the proteolytic conversion to PKM is catalyzed by calpain, a calcium-dependent kinase (Inoue *et al*, 1977, Suzuki *et al*, 1987). In addition to conversion to PKM, PKC can be extensively degraded within the cell, thereby losing all kinase activity. This process is termed down-regulation and often occurs as a result of prolonged activation of the kinase (reviewed by Kikkawa *et al*, 1989).

Much work is currently being done to assess the roles of the various PKC isoenzymes in specific cell types as well as the specific molecular mechanisms by which these roles are performed. A goal of this study is to gain further understanding of these aspects of protein kinase C in the parathyroid.

1.2 Calcium

1.2.1 Functions of calcium

Calcium plays an important role in a large number of biological processes. Bone, which contains the majority of calcium in the body, is essential for protection, posture and locomotion. The formation of complexes containing calcium and phosphate provides the rigid structure necessary for the functions of this tissue. Calcium in the extracellular fluid (ECF) is present in much smaller quantities but is important for conduction of nerve impulses and cellular communication. Within the cell, calcium is an important second messenger involved in a variety of signal transduction processes. Due to the importance of calcium in these as well as other processes, distribution of calcium within the body is tightly regulated. This regulation is accomplished by a homeostatic system

comprising three hormones: parathyroid hormone (PTH), calcitonin and 1,25-dihydroxyvitamin D.

1.2.2 Calcium homeostasis

Dietary calcium is distributed between bone, the extracellular fluid, and cells, or is excreted via the kidneys and intestine. For efficient cellular function, the calcium level in the ECF must be maintained within a relatively narrow range. In adult humans and cattle, this range is between 9 and 11 mg/100ml (Kronefeld *et al*, 1977), approximately half of which is bound to proteins and other anions. This results in a normal value of free, or ionized, calcium between 1.1 and 1.25 mM; although under certain conditions this level can vary between 0.5 and 2mM (Watson and Hanley, 1992). When the free calcium in the ECF drops below 1mM, parathyroid hormone (PTH) secretion increases. The increase in circulating levels of PTH causes: 1) an increase in the reabsorption of calcium from the distal tubules of the kidney, 2) increased resorption, or mobilization of calcium and phosphorus from the bone and 3) increased production of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). The $1,25(OH)_2D$ acts in conjunction with PTH to increase the level of calcium in the ECF by enhancing bone resorption and increasing calcium absorption in the intestine (Broadus, 1993). Under hypercalcemic conditions, PTH secretion decreases resulting in a net increase in calcium excretion. Further, the combined decrease in PTH levels and increased secretion of calcitonin are thought to decrease bone resorption. It is clear that PTH plays a major role in calcium homeostasis, and much work has been focused on the synthesis and secretion of PTH.

1.3 Parathyroid hormone

1.3.1 Structure of Bovine and Human PTH

Parathyroid hormone is a peptide hormone synthesized and secreted by the parathyroid gland. In 1970, the complete amino acid sequence of bovine PTH (bPTH) was elucidated (Brewer and Ronan, 1970). It is composed of 84 amino acids in a single polypeptide chain, and has a molecular weight of approximately 9600 daltons (Figure 1.2). Human parathyroid hormone (hPTH) also consists of 84 amino acids and is highly homologous to its bovine equivalent (Keutmann *et al*, 1978) (Figure 1.3). Although the sequence depicted in Figure 1.2 is threonine-free, two biologically active, threonine-containing isohormones of bovine PTH have been identified in addition to this one (Keutmann *et al*, 1971).

Early studies indicated that a fragment produced by dilute acid hydrolysis of bPTH, bPTH 1-29, retained most of the biological activity of the intact molecule (Keutmann *et al*, 1972). Currently, any PTH fragment lacking the amino-terminal 27 residues is considered to be devoid of PTH hypercalcemic, phosphaturic and hypocalciuric activity (Mallette and Gagel, 1993). Indeed, receptor binding and activation of second messenger systems within target cells requires the amino terminal 27 residues (Nutt *et al*, 1990). PTH-related peptide (PTHrP), a peptide secreted by a variety of tissues, binds the same receptor in target cells (Burtis, 1993). Circular dichroism and 2-dimensional proton nuclear magnetic resonance studies have been performed to analyze the secondary structure of hPTH (1-34). In one such study, the structure of this fragment in trifluoroethanol and dodecylphosphocholine micelles, thought to resemble the biological

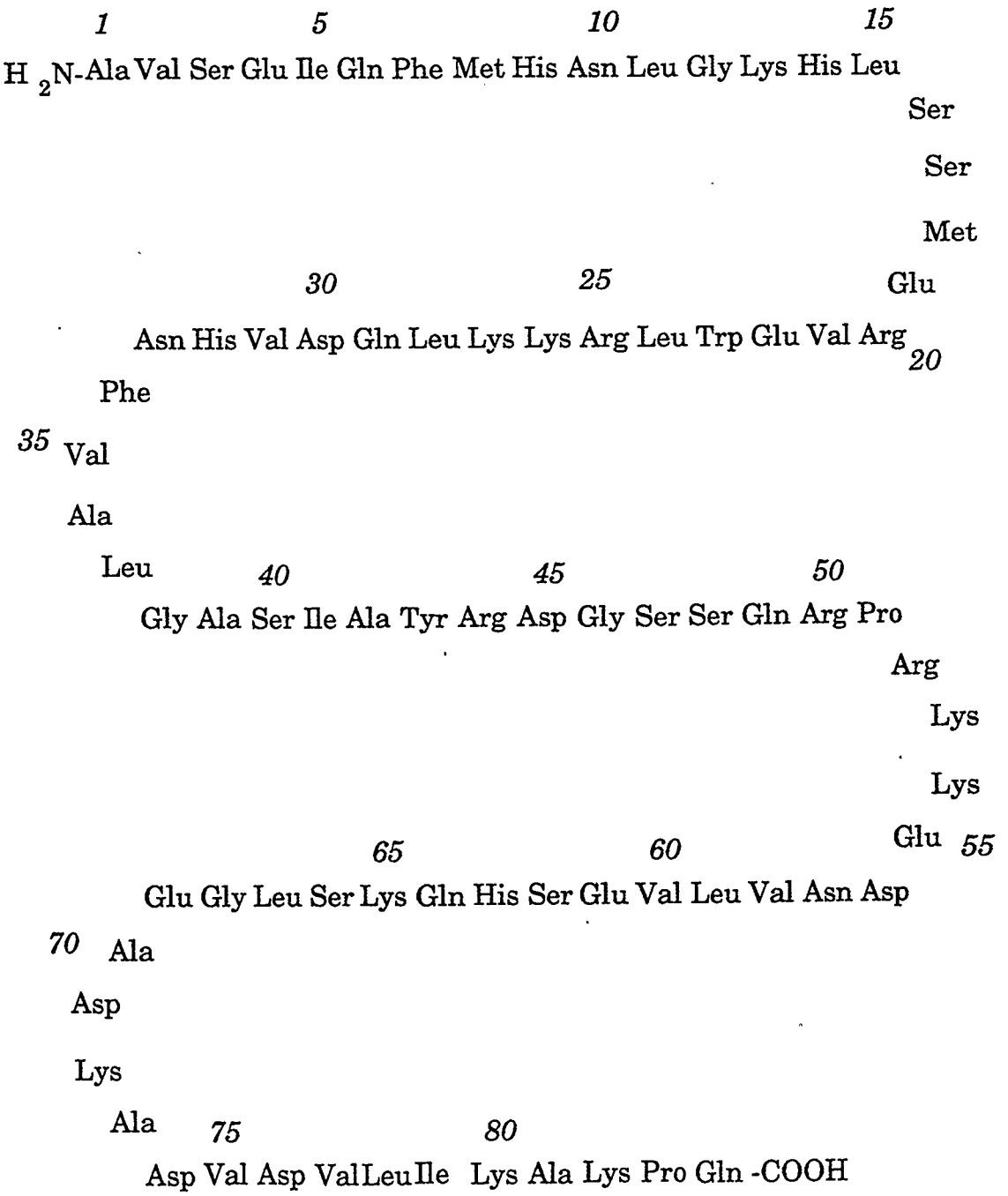

Figure 1.2 The amino acid sequence of bovine parathyroid hormone. Three letter codes are used to indicate specific amino acid residues.

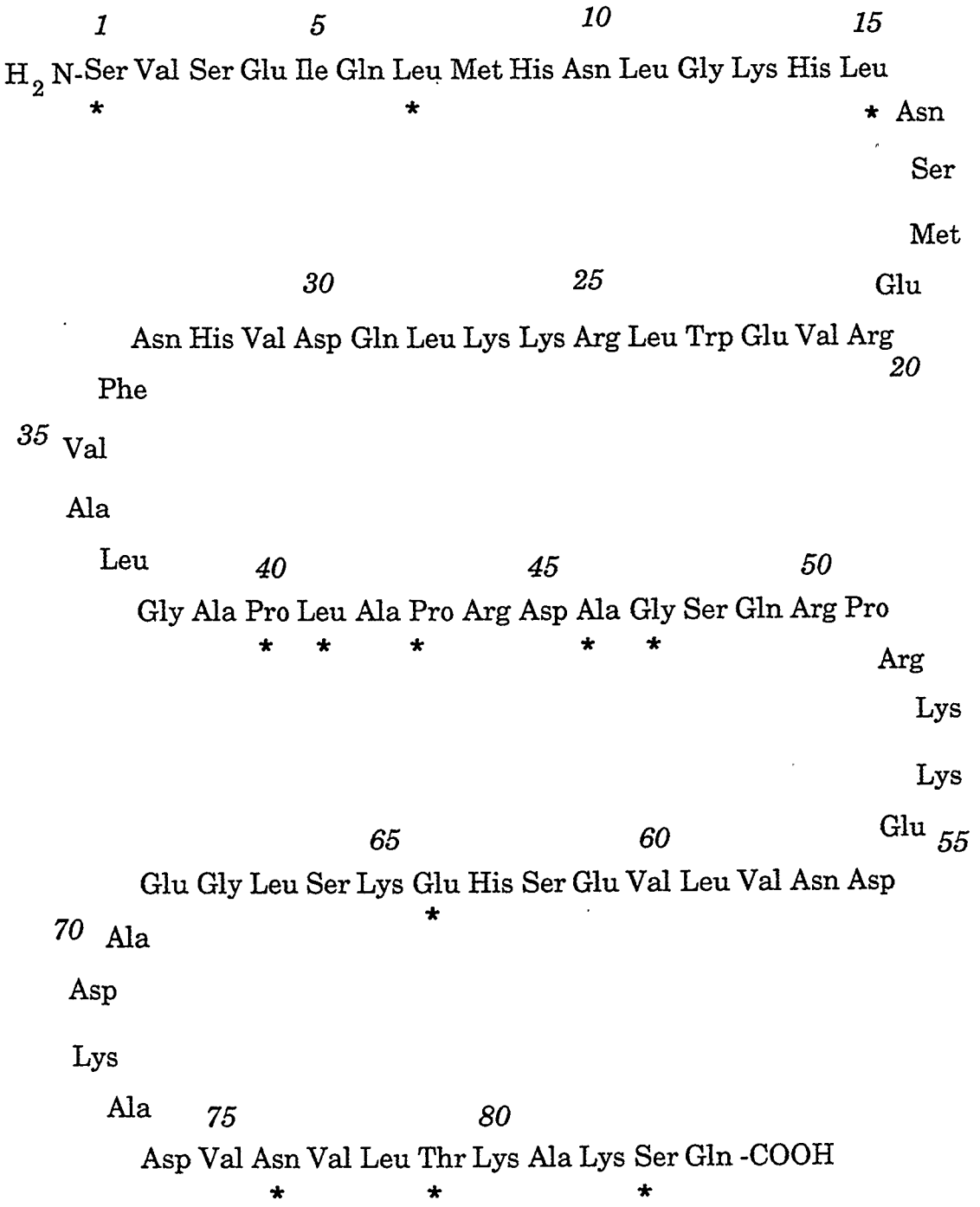

Figure 1.3 The amino acid sequence of human parathyroid hormone. Three letter codes are used to indicate specific amino acid residues. Asterisks (*) indicate residues which differ between human and bovine PTH.

membrane environment, was examined (Strickland *et al*, 1993). This study revealed that hPTH (1-34) consists of two alpha-helical regions, spanning between residues 3-12 and 17-26. It is interesting to note that the two amino-terminal residues appear to have random structure in this environment but are the specific determinants for activation of second messenger systems in target cells (Nutt *et al*, 1990). PTH residues 2-34 bind the PTH / PTHrP receptor with high affinity. However, PTHrP is homologous with PTH only in 8 of the first 13 residues, but binds the receptor with similar affinity (Strewler and Nissenson, 1993). In light of this finding, it is thought that alpha helical secondary structure shared by both these molecules in the amino-terminal 30 residues is a key determinant for receptor binding (Strewler and Nissenson, 1993).

Although the amino terminus contains the majority of the biological activity of PTH, some studies have indicated a role for the mid (44-68) and carboxyl-terminal (69-84) regions in PTH activity. It is possible that these regions of PTH alter the affinity of the binding of the amino-terminal region to the receptor (Demay *et al*, 1985), or increase the half-life of the molecule in the circulation (Martin *et al*, 1978). There is some evidence that the carboxyl terminal region of PTH binds a different receptor on cultured bone cells (McKee and Murray, 1985), and that this binding stimulates different pathways than the binding of the amino terminal fragment or intact PTH (Murray *et al*, 1991, Sutherland *et al*, 1994). Although they are not considered to be of prime clinical importance, an understanding of the possible functions of PTH fragments lacking the amino terminal 27 residues is relevant to the study of parathyroid physiology because such fragments are produced and secreted by the parathyroid gland, whereas

PTH fragments containing the amino terminal region of PTH have not been detected (Flueck *et al*, 1977).

1.3.2 Synthesis of PTH

Parathyroid hormone mRNA is constitutively synthesized in the parathyroid gland. Several factors have been identified as potential modulators of PTH gene expression (Watson and Hanley, 1992). These include vitamin D, which has been shown to decrease PTH gene transcription over a period of 24-48 hours (Silver *et al*, 1985). Other factors are proposed to act as modulators of PTH gene expression due to the presence of their response elements in the 5' flanking region of the PTH gene. These include cyclic adenosine monophosphate (cAMP) (Rupp *et al*, 1990), and glucocorticoids (Reis *et al*, 1990). Additionally, PTH mRNA production, stability and translation into protein may be dependent on extracellular calcium levels (Russel *et al*, 1983, Okazaki *et al*, 1991, Hawa *et al*, 1993).

The direct translation product of PTH mRNA, preproPTH, is a peptide of 115 amino acids (Kemper *et al*, 1974, Cohn and MacGregor, 1981). The amino terminal 25 residues of preproPTH comprise a highly hydrophobic region which acts as a signal sequence for ribosome association with the endoplasmic reticulum and insertion of the growing polypeptide into the cisternal space (reviewed by Cohn and MacGregor, 1981). PreproPTH exists only transiently and, when found in parathyroid cell homogenates, is almost exclusively associated with the membrane fraction (Haebener and Potts, 1979). The signal sequence of this PTH precursor is cleaved in the endoplasmic reticulum, and a 90 amino acid precursor, proPTH, is transported to the golgi apparatus. ProPTH, which

consists of 16 amino acids in the amino terminus in addition to the 84 amino acids of mature PTH, is easily isolated from parathyroid tissue (Kemper *et al*, 1972). ProPTH is then packaged in secretory vesicles and is converted by an integral membrane component into mature PTH (MacGregor *et al*, 1976, Morrissey and Cohn, 1979).

PTH is proteolytically cleaved within the parathyroid cell prior to secretion (Flueck *et al*, 1977, Hanley *et al*, 1978). Carboxyl fragments of PTH having amino termini at positions 43, 37, 34, 24 and 28 of intact PTH are secreted by the parathyroid, whereas amino terminal fragments appear to be extensively degraded within the cell (Morrissey *et al*, 1980, Cohn and MacGregor, 1981, MacGregor *et al*, 1983, MacGregor *et al*, 1986). The protease responsible for PTH degradation has not been identified. Differential centrifugation of parathyroid cell homogenates indicates that PTH fragments may reside in different cellular locations in comparison to intact PTH (MacGregor *et al*, 1983). Although secreted PTH fragments do not correspond to those produced by *in vitro* digestion with cathepsin B or D (MacGregor *et al*, 1986), inhibitors of lysosomal degradation increase the secretion of intact PTH (MacGregor and Bansal, 1989). The proportion of PTH fragments, relative to intact PTH, secreted by parathyroid increases with increased extracellular calcium (Hanley *et al*, 1978, Mayer *et al*, 1978, Hanley and Ayer, 1986). This may indicate that a calcium-dependent protease is responsible for PTH degradation (Reviewed by Watson and Hanley, 1992). Two calcium-activated neutral proteases, micro-calpain and milli-calpain, have been identified in bovine parathyroid cell extracts (Watson *et al*, 1995). Micro-calpain cleaves PTH *in vitro* to produce fragments which appear similar to those generated by the parathyroid; as

determined by reverse-phase high performance liquid chromatography (HPLC) (Watson *et al*, 1995). Calpains are of particular interest because the phosphorylation state of substrate proteins may alter their susceptibility to proteolysis by these enzymes (Rogers *et al*, 1986, Wang *et al*, 1989). Since PTH is phosphorylated *in vivo*, it is possible that phosphorylation alters the subsequent proteolysis of this hormone (Rabbani *et al*, 1984). The regulation of PTH degradation is probably an important aspect of parathyroid secretory response, because it is a route by which secretion of intact PTH can be modulated (reviewed by Watson and Hanley, 1992).

1.4 Factors affecting the secretion of PTH

Although changes in gene expression affect PTH synthesis, these changes occur over the long-term, and will not be described here. PTH is synthesized at a constant rate in the parathyroid. Even under physiological conditions which cause maximal inhibition of secretion, a constant baseline secretion of PTH occurs. Regulation of secretion in a short time frame (minutes to hours) can occur by alterations in storage, secretory processes, or degradation. Factors affecting PTH secretion include β–adrenergic agonists, cations, and pharmacological modulators of protein kinase C.

1.4.1 Effects of alterations in intracellular cAMP on PTH secretion

β adrenergic agonists, such as isoproterinol and epinephrine cause a rapid, but brief, increase in PTH secretion (Brown *et al*, 1977, Mayer *et al*, 1979). This increase in secretion is mediated by an increase in cellular levels of cyclic adenosine monophosphate (cAMP), and can be elicited by cAMP analogs such as dibutyril-cAMP (Brown *et al*, 1978a, Morrissey and Cohn, 1979a). Coupling of the β adrenergic receptor to G_s, a GTP-binding

protein which stimulates adenylate cyclase upon receptor occupation, has been established in many cell types (reviewed in Gennis, 1989). Furthermore, agents which bind receptors on the parathyroid cell surface linked to G_i, a GTP binding protein which inhibits adenylate cyclase, result in a decrease in PTH secretion (Brown, 1978b).

Increased secretion of PTH in response to increased cellular cAMP levels is not dependent on protein synthesis (Hanley *et al*, 1985). Further understanding of this phenomenon is provided by pulse-chase studies of PTH secretion (Morrissey and Cohn, 1979b). These studies indicated that, whereas newly synthesized PTH was constitutively secreted, PTH secreted in response to β adrenergic stimulation was derived from an older, or stored pool of PTH. These studies indicate that two pools of PTH are present in the parathyroid cell, one consisting of newly synthesized PTH and another, cAMP-accessible, storage pool (reviewed by Cohn and MacGregor, 1981). Because the storage pool is limited, agents which affect cellular cAMP levels are probably not prime regulators of sustained parathyroid secretory responses (reviewed by Watson and Hanley, 1992).

1.4.2 Calcium regulation of PTH secretion

Extracellular concentrations of calcium are the primary physiological regulators of PTH secretion (reviewed by Brown, 1991). Unlike many secretory cells, parathyroid cells decrease secretion in response to increased extracellular calcium (Ca_e). Physiologically, this represents a negative feedback control mechanism, whereby increased extracellular calcium levels decrease the secretion of this calciotropic hormone. Historically however, the parathyroid secretory response to Ca_e has presented a challenge to the understanding of secretory mechanisms in

general. In many secretory cells, decreases in extracellular calcium result in an increase in intracellular free calcium (Ca_i) and a coincident increase in secretion. In the parathyroid, intracellular calcium levels parallel those of extracellular calcium. Furthermore, increases in the levels of calcium cause increased secretion of PTH fragments and decreased overall PTH secretion.

Elevated Ca_e levels cause a decrease in intracellular levels of cAMP, however, the contribution of this effect is probably insignificant in the overall regulation of PTH secretion by calcium due to the relatively small PTH pool accessible by this second messenger (reviewed by Brown, 1991).

The classical stimulus-secretion coupling theory, based on the study of several secretory cells, suggests that as Ca_i increases, secretion increases (Douglas, 1968). Much work has been done to identify the mechanisms by which the parathyroid secretory response differs from other secretory cells. One study revealed that at subphysiological calcium levels (<10nM), PTH secretion is inhibited. As extracellular calcium increases, PTH secretion increases up to Ca_e of 1mM, but subsequently decreases at higher Ca_e levels (Nygren *et al*, 1987). Ca_i, measured with the fluorescent dye quin 2, paralleled the extracellular calcium levels. Maximal secretion at 1 mM Ca_e corresponded to 200 nM Ca_i. Thus secretion did display a positive correlation with Ca_i, albeit over a subphysiological range of Ca_i.

Experiments performed with parathyroid cells, which were permeablized to calcium and other small molecules by electric shock, also indicated classical stimulus-secretion coupling, but over a supraphysiological range of calcium (Oetting *et al*, 1987). In these studies

PTH secretion increased between 2 uM and 400 uM calcium, above which secretion decreased. Although both studies reveal aspects of classical stimulus-secretion coupling in the parathyroid, neither is relevant within the ranges of calcium observed physiologically.

Further work to characterize the unusual secretory response of the parathyroid focuses on the molecular mechanisms by which extracellular levels of calcium are sensed by the parathyroid and the biochemical pathways by which this extracellular calcium alters secretion.

1.4.3 The parathyroid calcium sensor

The unusual ability of the parathyroid to respond directly and primarily to extracellular calcium levels indicates the existence of some specialized mechanism by which this cell type senses calcium (Reviewed by Brown *et al*, 1990, Brown, 1991, and Pocotte *et al*, 1991). Other cations have similar effects on PTH secretion and Ca_i to those of Ca_e. These include divalent and trivalent cations, such as Mg^{2+}, La^{3+}, and Gd^{3+}, as well as polyvalent cations such as neomycin (Morrissey and Cohn, 1978, Brown *et al*, 1992, Racke and Nemeth, 1993a, Ridefelt *et al*, 1992). These observations suggest that parathyroid cells sense extracellular calcium levels via a cation specific receptor rather than a calcium channel. Several experimental approaches suggest that the parathyroid calcium sensor is a cell surface glycoprotein. Treatment of cells with concanavalin A, a lectin, blunts the effects of extracellular calcium on parathyroid secretion, indicating that the sensor contains carbohydrate moieties (Brown *et al*, 1992). Trypsin treatment of intact parathyroid cells also has similar effects on this calcium-sensitivity, indicating that a cell-surface protein mediates

calcium-responsiveness (Muff and Fischer, 1989). Monoclonal antibodies generated against intact cells from human parathyroid tissue recognize a high molecular weight glycoprotein on membranes of intact parathyroid cells (Juhlin *et al*, 1987). In addition a few other tissues which appear to display calcium-sensing abilities are recognized by these antibodies (Juhlin *et al*, 1990, Ackerstrom *et al*, 1991). Further, treatment of calcium-responsive cells with these antibodies uncouples their typical cellular responses to extracellular calcium (Hellman *et al*, 1993). Although these studies reveal that calcium is sensed by a glycoprotein on the cell surface, the structure of this putative receptor and its specific mode of transducing signals across the parathyroid cell membrane have not been determined.

Recently, a calcium-sensing receptor was cloned from a bovine parathyroid cDNA library (Brown *et al*, 1993). This receptor shares homology with the G-protein-coupled receptor superfamily, contains seven putative membrane spanning helices, and has several potential N-linked glycosylation sites, as well as PKC phosphorylation sites. When expressed in *Xenopus laevis* oocytes the bovine calcium receptor couples with a pertusis toxin-insensitive GTP-binding protein. Activation of the receptor in this system by calcium, as well as other di-, tri-, and poly- valent cations results in an increase in phospholipase C (PLC) activity via this G-protein (Brown *et al*, 1993). The properties of this receptor correlate well with what is currently known about the signal transduction pathways in parathyroid cells.

1.4.4 Signal transduction pathways in the parathyroid in response to extracellular calcium

Although the exact cellular mechanisms by which the parathyroid transduces signals about extracellular calcium levels into a secretory response are not currently known, several putative second messengers have been identified.

It is well documented that intracellular calcium levels increase in response to high extracellular calcium in the parathyroid. This calcium originates from two pools (Racke and Nemeth, 1993a and 1993b). Rapid, but transient, increases in Ca_i initially arise from an intracellular storage pool of sequestered calcium. This is followed by a steady influx of calcium from the extracellular fluid. In many cell types the former intracellular pool of calcium is released into the cytoplasm by an increase in cellular levels of inositol 1,4,5-trisphosphate (IP_3) (Shears, 1991).

IP_3 is a well known second messenger. In many signal transduction pathways, IP_3 accumulation is stimulated by activation of phosphatidylinositol-specific PLC enzymes. These PLCs hydrolyze phosphoinositides in cellular membranes producing diacylglycerol and soluble inositol phosphates, such as IP_3. In the parathyroid levels of IP_3 increase in response to elevated Ca_e (Shoback *et al*, 1988, Racke and Nemeth, 1993a) and to other di-, tri- and poly-valent cations (Brown *et al*, 1990). Evidence that this IP_3 is a cause, rather than the result of increases in intracellular calcium comes from studies in which divalent-cation ionophores were used to increase Ca_i (Shoback *et al*, 1988). In these studies IP_3 levels were not increased by artificially increasing intracellular calcium. The high Ca_e-elicited increase in IP_3 and corresponding decrease

in PTH secretion is mimicked by fluoride (Shoback and McGhee, 1988, Chen *et al*, 1989, Brown *et al*, 1990). Fluoride, in conjunction with trace quantities of aluminum, provides a route by which G-protein linked signaling pathways can be activated in the absence of ligand-receptor binding (Gennis, 1989). Furthermore, increased phosphoinositide hydrolysis in response to increased Ca_e is not inhibited by pertusis toxin treatment of parathyroid cells (Brown *et al*, 1990). This is important because pertusis toxin sensitivity can be used to distinguish between two different G-proteins, G_q and $G_{\alpha 11}$, known to activate PLC (Martin, 1991, Martin *et al*, 1992). Taken together, these findings suggest the possibility that a signal transduction pathway involving a G_q-like G-protein linked to phospholipase C activation, proximal to an increase in Ca_i, is part of the parathyroid response to elevated Ca_e.

In addition to IP_3, other phosphorylated forms of inositol accumulate in the parathyroid in response to elevated Ca_e (Brown *et al*, 1987). These include other characteristic products of phosphatidylinositol-specific PLC activity, such as inositol monophosphates and inositol bisphosphates (Nishizuka, 1992, Hawkins *et al*, 1989). Additionally, inositol 1,3,4,5 tetrakisphosphate IP_4, a downstream product of phosphatidylinositol 3'-kinase (PI3'-kinase) may increase at high Ca_e (Hawkins *et al*, 1989).

It is possible that each inositol phosphate performs a specific role within the cell, and would thus bind different receptors. Membrane binding studies indicate that IP_3 binds with high affinity to a single class of receptors in parathyroid cell membranes, as would be expected from studies of IP_3 receptors in other cells (Enyedi *et al*, 1989, Shears, 1991). IP_4 binds two membrane sites, each thermodynamically and kinetically

different from the IP_3 site (Enyedi *et al*, 1989), suggesting that IP_4 may perform distinct roles in the parathyroid calcium response. It has been suggested that IP_4 mediates the sustained influx of calcium into the parathyroid cell after the IP_3-mediated release of calcium from intracellular stores (Enyedi *et al*, 1989, Hawkins *et al*, 1990, Racke and Nemeth, 1993a).

In addition to inositol phosphates and calcium, at least two products of phospholipid metabolism are observed to accumulate in the parathyroid in response to elevated Ca_e. These are arachidonic acid and diacylglycerol (Bourdeau *et al*, 1992, Kifor and Brown, 1988). Two generalized enzyme systems, phospholipase A_2 (PLA_2) and diacylglycerol lipase are known to produce arachidonic acid in the cell. Diacylglycerol lipase hydrolyses fatty acids from DAG producing monoacylglycerol. PLA_2 enzymes cleave fatty acids from the sn-2 position of phospholipids, liberating predominantly cis-unsaturated fatty acids and the corresponding lysophospholipid. Several PLA_2 enzymes have been identified, and they differ in substrate specificity and activation. Activators of protein kinase C stimulate PLA_2 activity in several cell types, although it is uncertain whether this occurs directly or as a result of PKC activation (reviewed by Nishizuka, 1992). Studies using PLA_2 inhibitors indicate that this enzyme may be responsible for the production of arachidonic acid in the parathyroid cell exposed to high Ca_e, as high calcium-induced inhibition of PTH secretion is reversed by these PLA_2 inhibitors (Bourdeau *et al*, 1992). Arachidonic acid, as well as metabolites of arachidonic acid (via the 12- and 15- lipoxygenase pathway), inhibit secretion when added to parathyroid cells at low Ca_e (Bourdeau *et al*, 1992, Bourdeau *et al* 1994). Although the precise targets of these metabolites

remain to be elucidated, the information available suggests that these moieties may mediate the parathyroid secretory response to elevated Ca_e.

Diacylglycerol may also play a role in the parathyroid response to calcium. This metabolite is a product of PLC-mediated hydrolysis of phospholipids, and is a well known endogenous activator of protein kinase C (Reviewed by Nishizuka, 1992). The cellular levels of DAG increase in association with high calcium-induced inhibition of PTH secretion (Kifor and Brown, 1988). The increased cellular levels of calcium, arachidonic acid (and possibly other cis-unsaturated fatty acids, produced via PLA_2 activity), and DAG, associated with elevated extracellular calcium suggests that PKC would be activated in the parathyroid under these conditions. One implication is that PKC activation may be associated with increased degradation and decreased overall secretion of PTH. However, studies on secretion in other cell types, as well as the parathyroid do not support this implication.

In several cell types it has been established that activation of PKC is necessary for exocytosis (Reviewed by Kikkawa *et al*, 1987, Sarafian, 1991, Morgan *et al*, 1993). The PKC-activating cellular environment and the inhibition of secretion at high calcium in the parathyroid is inconsistent with this generalization and has been examined by several means. Studies of PKC activation, as determined by membrane association of phospholipid- and calcium-dependent PKC activity in parathyroid cells, indicate that PKC is activated to a greater extent at low calcium than at high calcium (Kobayashi *et al*, 1988, Morrissey, 1988). Based on these findings it would seem that the parathyroid secretory response is similar to other cells in that increased PKC activation coincides with states of increased secretion.

However, PKC activation appears to occur under cellular conditions which do not promote activation, and decreases under cellular conditions which are thought to activate PKC. Several studies using activators and inhibitors of PKC have been employed to examine this inconsistency.

1.4.5 Effects of PKC activators and inhibitors on PTH secretion

The ability of phorbol esters to activate classical and novel PKCs has been demonstrated *in vitro* as well as *in vivo* (Nishizuka, 1992 and Hug and Sarre, 1993). Several studies have found that biologically active phorbol esters such as phorbol myristate acetate reverse the inhibition of PTH secretion observed at high calcium (Brown *et al*, 1984, Muff and Fischer, 1986, Nemeth *et al*, 1986, Morrissey 1988, Membreno *et al*, 1989, Shoback and Chen, 1990, Tanguay *et al*, 1991, Ridefelt *et al*, 1992, Clarke *et al*, 1993, Racke and Nemeth, 1993a). Thus PKC activation by phorbol esters results in an increase in PTH secretion at high Ca_e, indicating that secretion may be mediated by PKC. However, in addition to increasing secretion at high calcium, phorbol ester treatment blunts other high Ca_e-induced cellular responses in the parathyroid. These include inositol phosphate accumulation (Shoback and Chen, 1990) and increased intracellular calcium levels (Muff and Fischer, 1986, Nemeth and Scarpa, 1986, Racke and Nemeth, 1993a). Furthermore, studies indicate that at low Ca_e several phorbol esters decrease PTH secretion (Membreno *et al*, 1989, Tanguay *et al*, 1991, Racke and Nemeth, 1993a), and increase PTH degradation (Tanguay *et al*, 1991).

Studies with other PKC activators are consistent with the data derived from PMA experiments at high calcium (Membreno *et al*, 1989, Watson *et al*, 1992, Racke and Nemeth, 1993a). At low calcium, however,

the results varied with the PKC activator used. Bryostatin and dioctanoyl glycerol (Membreno *et al*, 1989), as well as mezerein and indolactam V (Racke and Nemeth, 1993a) mimic the inhibitory actions of PMA on low Ca_e-stimulated secretion. Two other PKC activators, SC-9 and SC-10, had no effect on the low calcium-induced secretory response (Watson *et al*, 1992). It is possible that the differences in the effects observed are due to activation of other enzymes or differences in the specific PKC isoenzyme(s) affected by these activators.

Several PKC inhibitors have been used to assess the role of PKC in the parathyroid responses to extracellular calcium levels. One inhibitor of PKC, H-7, decreases PTH release at low Ca_e (Watson *et al*, 1992, Clarke *et al*, 1993). These studies support the idea that PKC is involved in the secretion of PTH at low calcium, because this secretion is inhibited by this PKC inhibitor. However, H-7 appears to be more potent at inhibiting cPKCs than nPKCs (Oudinet *et al*, 1992), and may inhibit other protein kinases (Wilkinson and Hallam, 1994). Furthermore, staurosporine, a potent inhibitor of both classical and novel PKCs (Wilkinson and Hallam, 1994) has no effect on PTH secretion at either high or low Ca_e (Racke and Nemeth, 1993b). Both H-7 and staurosporine were able to reverse the actions of the PKC activators, including PMA, on overall PTH secretion (Watson *et al*, 1992, Racke and Nemeth, 1993b). However, H-7 was not effective in reversing the PMA-induced degradation of PTH at low Ca_e (Watson *et al*, 1992).

1.5 Objectives

It is evident from the preceding sections that PKC plays an important role in a diverse range of cellular functions, and that individual isoenzymes

may perform specialized tasks within a given cell. Due to the diversity of PKC isoenzymes identified to date, a primary goal of this study is to identify the specific PKC isoenzymes expressed in the parathyroid. This may then aid in the understanding of PKC isoenzyme specialization as well as parathyroid cell regulation.

Another goal of this study is to examine the conditions under which the classical PKC isoenzymes are activated in relation to the secretory state of the parathyroid cell. The intracellular conditions of the parathyroid cell under the low calcium-stimulated secretory state would not appear to promote the activation of the cPKCs, yet these isoenzymes have been identified as important components of the secretory response. Thus, it is hoped that the study of cPKC isoenzyme specialization will allow greater understanding of the regulation of the individual isoenzymes as well as their involvement in the secretory process of the parathyroid.

Protein kinase C has been identified as an important factor in the regulation of both the secretion and degradation of PTH. Since phosphorylation of other proteins is known to alter their proteolytic degradation, and PTH is phosphorylated within the parathyroid, another goal of this study is to demonstrate that PKC phosphorylates PTH *in vitro*. It is hoped that identification of the PKC-mediated phosphorylation site in PTH from both bovine and human will aid in future studies of PTH degradation and secretion.

Methods and materials

2.1 Samples

Western blots were performed on total parathyroid cell preparations; treated parathyroid tissue (soluble and particulate fractions); and parathyroid cell soluble, particulate and triton-insoluble fractions.

2.1.2 Culture media

1) Wash medium: Hank's Balanced Salts solution (GIBCO BRL, Burlington, Ont., Canada), containing 15 mM HEPES, 4.2 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 0.8 mM $MgCl_2$, pH 7.4 containing 10 mg/ml Gentamicin Sulphate, 10,000 u/ml Penicillin G, 10 mg/ml Streptomycin Sulphate, 10 mg/ml amphotericin B. 2) Digestion medium: Waymouth MB 752/1 medium (GIBCO BRL) containing 15 mM HEPES, 4.2 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 0.75 mM $MgCl_2$, 10 mg/ml Gentamicin Sulphate, 10,000 u/ml Penicillin G, 10 mg/ml Streptomycin Sulphate, 10 mg/ml Amphotericin B, pH 7.4. 3) Culture medium: Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO BRL) containing 15 mM HEPES, 44 mM $NaHCO_3$, 1.25 mM $CaCl_2$, 0.75 mM $MgCl_2$, 0.87 uM bovine insulin (Sigma Chemical Co., St. Louis, MO., USA), 64 nM bovine transferrin (Sigma), 10 mg/ml Gentamicin Sulphate, 10 mg/ml Amphotericin B, pH 7.4.

2.1.3 Preparation of parathyroid tissue

Superior parathyroid glands (approximately 80) were removed from adult cows (*Bos bostratum*) within 15 minutes of slaughter. They were immediately placed in ice-cold wash medium and transported to the lab for processing. The glands were rinsed briefly with a 75 uM NaCl, 30% ethanol solution, followed by three rinses in fresh wash medium. Excess fat was trimmed from the glands, which were then minced using a Stadie-Riggs

tissue slicer (Thomas Scientific Inc., Swedesboro, NJ, USA). The tissue was placed in fresh wash medium and further minced using surgical scissors. Fat released by this process floated to the surface and was aspirated off. When no more fat could be liberated, the minced tissue was centrifuged at 12,000 rpm for 10 min. The pellet was then either collagenase digested to yield individual cells or resuspended in wash medium, centrifuged and used for treatment with calcium and PMA.

2.1.4 Isolation of bovine parathyroid cells

Minced parathyroid tissue was transferred to 80 ml digestion medium. Collagenase (400-600 U/ml; Yakult, Tokyo, Japan) and 200 ug/ml deoxyribonuclease II (Sigma) were added and the tissue was digested for 6-8 hr in a shaking water bath at 37°C, under 1 atmosphere of Carbogen gas (Medigas, Calgary, Ab., Canada). At thirty minute intervals, the tissue was aspirated using a 10 ml serological pipette fitted to a 50 ml syringe to further disperse the tissue. Light microscopy was utilized to monitor the extent of digestion. When clumps of 6-8 cells were visible, papain (Boehringer Mannheim Biochemicals, Montreal, Que., Canada) was added to a concentration of 150ug/ml , and the digestion was continued for 1 hr. The cells were then centrifuged at 1000 rpm for 10 min in a Beckman TJ-6 centrifuge. The medium and a thin layer of red blood cells were gently aspirated off and replaced with culture medium. The centrifugation procedure was repeated 3 times to remove all red blood cells, each time resuspending the cell-containing pellet in fresh culture medium. The viability of the resuspended cells was determined by Trypan Blue exclusion, and typically exceeded 95%. The cells were then centrifuged once more and the pellet was processed for immunoblotting as described below.

2.1.5 Calcium and PMA treatments of bovine parathyroid tissue

Minced bovine parathyroid tissue was divided into four tubes containing 5% fetal calf serum, 0.8 mM $MgCl_2$-supplemented DMEM (GIBCO BRL), and either 0.5 or 2.0 mM calcium in the presence or absence of 1.6 uM PMA (Sigma). The tubes were shaken in a 37°C water bath under 1 atm pressure of Carbogen gas. Medium samples were taken at 0, 15, 30, and 45 minutes for PTH measurement by radioimmunoassay (RIA) using the guinea pig antibody GP-467, which has a detection preference for intact PTH (Hanley and Wellings, 1985). Immediately after the final collection of medium, the treated tissue was collected by centrifugation and separated into soluble and particulate fractions as described below.

2.2 Preparation of samples for immunoblotting

2.2.1 Protein assay

The protein content of samples used for immunoblotting was determined by a modification of the Lowry protein assay (Peterson, 1977). Bovine serum albumin (Sigma) was used for calibration.

2.2.2 Preparation of total parathyroid protein for immunoblotting

Parathyroid cells obtained from collagenase digestion of minced glands were homogenized in 2 volumes of 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.1 uM PMSF, 0.1 uM leupeptin (Sigma), pH 7.5. Hot SDS sample buffer (Laemmli, 1970) was added to the homogenate to give a final protein concentration of approximately 2 mg/ml. Samples were placed in a boiling water bath for 10 min, and frozen until used for SDS-PAGE.

2.2.3 Separation of soluble, particulate and triton-insoluble fractions for immunoblotting

Parathyroid cells obtained by collagenase digestion of fresh parathyroid tissue were homogenized in 2 volumes ice cold homogenization buffer (20 mM Tris, 5 mM EGTA, 2 mM EDTA, 1 uM PMSF, 2 ug/ml leupeptin, pH 7.5). The homogenate was centrifuged at 15,000 x *g* for 20 min and the supernatant was retained as the soluble fraction. Homogenization buffer containing 1% Triton X-100 was added to the pellet and the homogenization and centrifugation procedures were repeated. The supernatant and pellet from this step were retained as the particulate and triton insoluble fractions, respectively. Hot SDS sample buffer was added to each fraction to obtain a final protein concentration of 1-2 ug/ul, and the samples were placed in a boiling water bath for 10 min. Fresh bovine brain, rabbit lung, and rabbit biceps were also fractionated by this procedure to obtain control fractions for blotting.

2.3 SDS polyacrylamide gel electrophoresis and electroblotting

Electrophoresis was performed essentially as described by Laemmli (1970). Twenty micrograms of prepared protein was loaded per well in parallel with biotinylated high molecular weight markers (Bio-Rad, Richmond, CA., USA) and separated on a 7.5% polyacrylamide (7.5%T, 3.25%C) SDS minigel (Bio-Rad). Electrophoresis was carried out at 160 mV for 50 minutes. The separated proteins were electrophoretically transferred onto methanol-activated polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Marlborough, MA., USA) using a Trans-Blot tank apparatus (Bio-Rad). Blotting was carried out in transfer buffer (20% methanol, 25 mM Tris, 192 mM glycine, pH 8.3) for 4 hr at 80 mV. To

visualize the extent of protein transfer, the membranes were stained for 1 min with 1% fast green in a 10% acetic acid- 50% methanol- destain solution. Destaining was accomplished by three 2 min washes in destain. Strips containing blotted molecular weight markers were excised. The membranes were allowed to dry and stored for later immunodetection.

2.4 Immunodetection

2.4.1 Antisera

Five isoenzyme-specific antisera to PKC were used in this study. Rabbit anti-PKC α was purchased from Calbiochem (San Diego, CA., USA). This antiserum was produced by innoculation with a peptide corresponding to residues 313-326 of rabbit PKC α in bovine serum albumin carrier protein, and displayed less than 0.1% cross-reactivity with other PKC isoenzymes. Because this antiserum was not affinity purified, the blocking solution used with this antiserum was 10% goat serum in 0.05% Tween Tris Buffered Saline (TTBS). The antiserum was used at a dilution of 1:1000 in this blocking solution. Affinity-purified antiserum to the PKC β fragment (313-329) was purchased from Boehringer Manneheim (Montreal, Que., Canada) and used at a dilution of 1:500 in 5% non-fat milk in TTBS blocking solution. Affinity-purified antisera to PKC δ (region 657-673), ε (region 722-736), η (region 669-683), θ (region 690-707), and ζ (region 574-592) were purchased from Santa-Cruz Biotechnologies (Santa Cruz, CA. USA). These were used at a dilution of 1:100 in 5% non-fat milk, TTBS blocking solution. The secondary antibody used in all immunoblotting procedures was horseradish peroxidase-conjugated goat antirabbit (Amersham, Oakville, Ont., Canada), and was used at a dilution of 1:2000 in the same blocking solution used for the primary antibody.

2.4.2 Immunodetection of PKCs on PVDF membranes

Dried, blotted PVDF membranes were wetted with methanol, rinsed three times with distilled water, and placed in blocking solution at 4°C overnight. The membranes were then incubated for 4 hr in diluted primary antibody solution. These were washed 3 times with TTBS, then incubated in diluted secondary antibody solution. The membranes were again washed 3 times with TTBS. The development of the immunoblots was accomplished by immersing the membranes in freshly prepared enhanced chemiluminescence reagents (ECL, Amersham) for one minute followed by 1-5 min exposure on Kodak XK film.

2.4.3 Detection of molecular weight standards

Dried strips containing the transferred molecular weight markers were wetted and incubated in blocking solution at 4°C overnight. Streptavidin-conjugated horseradish peroxidase was then added to the blocking solution at a dilution of 1:25,000. After 30 min of incubation in this solution, the strip was removed and washed 3 times with TTBS. This was then immersed in freshly prepared enhanced chemiluminescence reagents (ECL, Amersham) for one minute and aligned with the corresponding immunodetected membrane prior to exposure to the film.

2.4.4 Identification of immunospecific bands

Immunospecific bands for each isoenzyme were identified by their disappearance when preadsorbed primary antiserum was used instead of the corresponding primary antisera in the immunodetection procedure. For PKC α, the diluted primary antiserum was incubated overnight at 4°C with approximately 1 ug/ml PKC purified from bovine brain (the purification is described in the section 2.5). For the other antisera,

preadsorption was carried out using the commercially available antigens used to produce the antisera, at the recomended ratio to the diluted primary antibody.

2.5 Purification of PKC

2.5.1 Buffers

1) Homogenization buffer consisted of 10 mM Tris pH 7.5, 0.32 M sucrose, 10 mM EGTA, 4 mM $MgCl_2$, 1 mM DTT, 1 uM PMSF, 2 ug/ml leupeptin. 2) DEAE buffer contained 10 mM Tris pH 7.5, 0.1 mM EGTA, 1 uM DTT, 1 uM PMSF, 2 ug/ml leupeptin. 3) Phenyl Sepharose buffer consisted of 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 uM PMSF, 2 ug/ml leupeptin. 4) Hydroxylapatite start buffer contained 10 mM potassium phosphate, 1 mM EGTA, pH7.5.

2.5.2 Determination of PKC activity

PKC activity was determined by measuring phosphate incorporation into the PKC-specific peptide substrate corresponding from an 11 amino acid sequence of myelin basic protein (MBP_{4-14}) (Sigma). One microliter of each column fraction was added to 20 ul of 20 mM Tris, pH7.5, 10 mM $MgCl_2$, 10 uM ATP (0.5-1uCi γ^{32}P-ATP/assay) (Amersham), and 50 ug/ml MBP_{4-14}, in the presence or absence of 5 mM $CaCl_2$, 25 ug/ml phosphatidylserine and 8 ug/ml diolein vesicles prepared by sonication. The phosphorylation reaction was carried out for 10min at 30°C, after which 15 ul of reaction mix was blotted onto Whatman P81 phosphocellulose paper (Fisher Scientific, Montreal, Que., Canada). The papers were then washed 3 times with 75 mM phosphoric acid, dried, and Cerenkov counted. To determine net PKC activity, the values obtained in the absence of calcium

and phospholipid vesicles were subtracted from those obtained in their presence.

2.5.3 DEAE and G-100 purification of PKC from bovine brain

Approximately 30 g of fresh bovine brain tissue was homogenized in three volumes of cold homogenization buffer. The homogenate was centrifuged at 100,000 x *g* for 90 min in a swinging bucket rotor, and the supernatant was adsorbed to 100 ml of swollen DEAE A-50 matrix (Pharmacia, Uppsala, Sweden). The matrix was then washed with three volumes of DEAE buffer and eluted with a 600 ml gradient of DEAE buffer containing 0 to 250 mM NaCl. Fractions of 5 ml were collected and assayed for PKC activity. Fractions eluting between 90 and 160 mM NaCl contained the majority of PKC activity. These were pooled and concentrated using an A 15 membrane on an Amicon concentrator (Oakville, Ont., Canada). The concentrate was then run on a 3.5 x 100 mm Sephadex G-100 (Pharmacia) column equilibrated with start buffer. Fractions eluting immediately after the void peak contained PKC activity and were pooled and further purified using Phenyl Sepharose chromatography.

2.5.4 DEAE purification of bovine parathyroid cell PKC

Approximately 1 x 10^8 bovine parathyroid cells obtained from collagenase digestion of minced glands were homogenized in 5 volumes ice-cold homogenization buffer. The homogenate was centrifuged at 100,000 x *g* for 1hr and the supernatant was adsorbed to DEAE A-50 (Pharmacia), equilibrated in DEAE buffer. After washing the beads with 3 volumes of DEAE buffer, they were poured into a column. A gradient of NaCl from 0 to 300 mM in DEAE buffer was used to elute the proteins. Fractions having PKC activity between 100 mM and 160 mM NaCl were pooled and either

further purified using Phenyl Sepharose chromatography or analysed for cPKC isoenzyme content using hydroxylapatite chromatography.

2.5.5 Phenyl Sepharose purification of PKC

Partially purified samples of PKC were brought to 2 M NaCl. This mixture was adsorbed to 10ml Phenyl Sepharose CL4B (Pharmacia) equilibrated with 2M NaCl Phenyl Sepharose start buffer. The matrix was then washed with three volumes each of 1.5 M, 1.0 M, 0.5 M, and 0.25 M NaCl in Phenyl Sepharose start buffer, prior to elution of the majority of PKC activity by 0 mM NaCl-containing start buffer.

2.5.6 Hydroxylapatite resolution of DEAE-purified PKCs

The pooled eluate from the DEAE purification of bovine parathyroid PKC was dialyzed against hydroxylapatite start buffer. This was then loaded onto a 5 ml hydroxylapatite (Bio-Rad) column and washed with 15 ml hydroxylapatite start buffer. The column was eluted with a gradient of 10 mM to 300 mM potassium phosphate, pH7.5 buffer containing 1 mM EGTA over 60 ml. Fractions of 1 ml were collected and assayed for PKC activity.

2.6 PTH phosphorylation

2.6.1 Time course of PTH phosphorylation and determination of phosphorylation stoichiometry for bovine PTH

Ten micrograms of bovine PTH (bPTH) (Bachem, Torrance, CA., USA) were phosphorylated in 500 ul of 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 1mM $CaCl_2$, 10 ug/ml leupeptin, 40 nmol ATP (20uCi γ^{32}P-ATP) solution containing 25 ug phosphatidylserine and 8 ug diolein, in vesicles prepared by sonication and added to the reaction mix. The reaction was initiated by addition of the phenyl sepharose-purified bovine brain PKC and incubation

at 30°C. Aliquots of 20 ul each were removed and placed in SDS gel electrophoresis buffer at 0, 10, 20, 50, and 75 minutes of reaction. These samples were run on 20% polyacrylamide SDS gels, as described earlier. Bands corresponding to PTH were identified by fast green staining for 30 min, followed by destaining overnight. These bands were excised and liquid scintillation counted.

2.6.2 Reverse phase HPLC

All reverse phase HPLC procedures were performed on a two pump LKB Bromma HPLC System (Fisher Scientific, Montreal, Que., Canada) attached to a C-18 uBondapak 8 x 100 mm Radialpak column (Waters Scientific, Mississauga, Ont., Canada). Solvents were filtered through a Millipore GVWP filter (Fisher Scientific) prior to use. Solvent A contained 0.08% trifluoroacetic acid (TFA) (Pierce, Rockford, IL, USA) and 0.0125% Acetic Acid (BDH Chemicals, Poole, Dorset, UK) in HPLC-grade water. Solvent B contained 0.08% TFA in 70% aqueous acetonitrile (HPLC grade) (BDH Chemicals). The solvents were delivered by the respective pumps A and B and linear gradients of increasing acetonitrile concentration were formed under the control of the solvent programmer unit. Absorbance was monitored at 214 nm. Fractions were collected in 1.5 ml microtubes and Cerenkov counted in an LKB Wallac Rack Beta II liquid scintillation counter (Cambridge, England) to determine the relative radioactivity in each fraction.

2.6.3 HPLC purification of phosphorylated bPTH

Larger quantities of bovine PTH were phosphorylated at a concentration of 20 ug/ml in the reaction mix described above for 60 minutes. Ten microliters of acetic acid was added per ml reaction mix and

this was then injected onto the C-18 uBondapak column equilibrated with HPLC solvent A. A discontinuous linear gradient of increasing HPLC solvent B was used to elute a single peak containing phosphorylated bPTH (P-bPTH).

2.6.4 Phospho-amino acid analysis of PKC-phosphorylated b PTH

The dried ^{32}P-labelled bPTH was subjected to 6 N HCl at 110^{o}C for two hours to hydrolyze the peptide bonds. The hydrolysate was blotted onto cellulose TLC plates (Kodak) and electrophoresed in parallel with P-Serine, P-Threonine and P-Tyrosine standards using an H_2O: acetic acid: pyridine (95:5:0.125) solvent system. The TLC plate was then autoradiographed and sprayed with ninhydrin to identify the migration of the standards.

2.6.5 Determination of the bPTH phosphate acceptor site

Purified ^{32}P-labelled P-bPTH mixture was resuspended in 100 ul of 100 mM Tris pH 6.8, 2 mM dithiothreitol and 1 mM EGTA buffer. *Staphylococcus aureus* strain V8 protease (Sigma) was added at a 1:5 molar ratio of protease to PTH and digestion was carried out for 18 hours at 30°C. Ten microliters of acetic acid were added and the digestion mix was injected onto the C-18 uBondapak column equilibrated with HPLC solvent A. A discontinuous linear gradient of increasing HPLC solvent B was used to elute the bound peptides. The two eluant peaks containing the majority of radioactivity were pooled, dried and sent for sequencing at the University of Calgary Protein Sequencing Facility. The phosphopeptides were covalently attached to a Sequelon-AA support (Millipore) and sequential Edman degradation was performed in a continuous flow reactor using an applied Biosystems 470A gas-phase sequencer. Radioactivity released in each

sequencing step was determined by Cerenkov counting. Additionally, the amino acid released in each cycle was identified on a C-18 Econosphere cartridge (Alltech) attached to a Beckman System Gold HPLC system, using norleucine as an internal standard.

2.6.6 Phosphorylation of human PTH

Forty micrograms of human PTH (hPTH) (Allelix, Mississauga, Ont. Canada) were phosphorylated in 2 ml of 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$,10 ug/ml leupeptin, 40 nmol ATP (20uCi γ^{32}P-ATP) solution containing 25 ug phosphatidylserine and 8 ug diolein vesicles prepared by sonication. The phosphorylation reaction was initiated by addition of the phenyl sepharose-purified bovine brain PKC and was allowed to proceed for 60 min at 30°C. Ten microliters of acetic acid was added and the reaction mix was injected onto the C-18 uBondapak column equilibrated with HPLC solvent A. A discontinuous linear gradient of increasing HPLC solvent B was used to elute the phosphorylated hPTH. This was dried down and resuspended in 100 ul of 100 mM Tris pH 6.8, 2 mM dithiothreitol and 1 mM EGTA buffer. *Staphylococcus aureus* strain V8 protease (Sigma) was added at a 1:5 molar ratio of protease to PTH and digestion was carried out for 8 hours at 30°C. The digestion mix was then purified on the C-18 uBondapak column. The eluant peak containing the majority of incorporated radioactivity was dried and sent for sequencing as described above.

Results

3.1 Identification of PKC isoenzymes in the bovine parathyroid

3.1.1 Classical PKC isoenzymes

The classical PKC isoenzymes, PKC α and β were detected in bovine parathyroid extracts by both immunological and chromatographic methods. Parathyroid proteins displaying PKC α- and PKC β- specific immunoreactivity had approximate molecular weights of 78 and 82 kilodaltons (kDa), respectively (see Figures 3.1A and 3.1B). Immunoreactive bands of similar molecular weights were also found in bovine brain soluble extracts, which are known to be enriched in both α and β PKC isoenzymes (Kikkawa *et al*, 1987). Preincubation of the isoenzyme-specific antibodies with purified protein kinase C from bovine brain or the control peptide used to generate the antibodies resulted in the inability to detect these bands in extracts from both brain and parathyroid, indicating that the 78 and 82 kDa bands are immunospecific for PKC α- and β-directed antibodies, respectively.

PKC α and PKC β were also detected by hydroxylapatite chromatography of partially purified bovine parathyroid PKC. Hydroxylapatite chromatography has frequently been used to separate the classical PKC isoenzymes (Huang *et al*, 1986). In this study, chromatography of DEAE-purified parathyroid cell homogenate produced two peaks of phospholipid- and calcium-dependent protein kinase C activity (see Figure 3.1C). The first peak, eluting at approximately 140 mM potassium phosphate was identified by dot blots to contain PKC β immunoreactivity. Furthermore, this is the characteristic elution position of PKC β from bovine brain. The second peak of PKC activity roughly

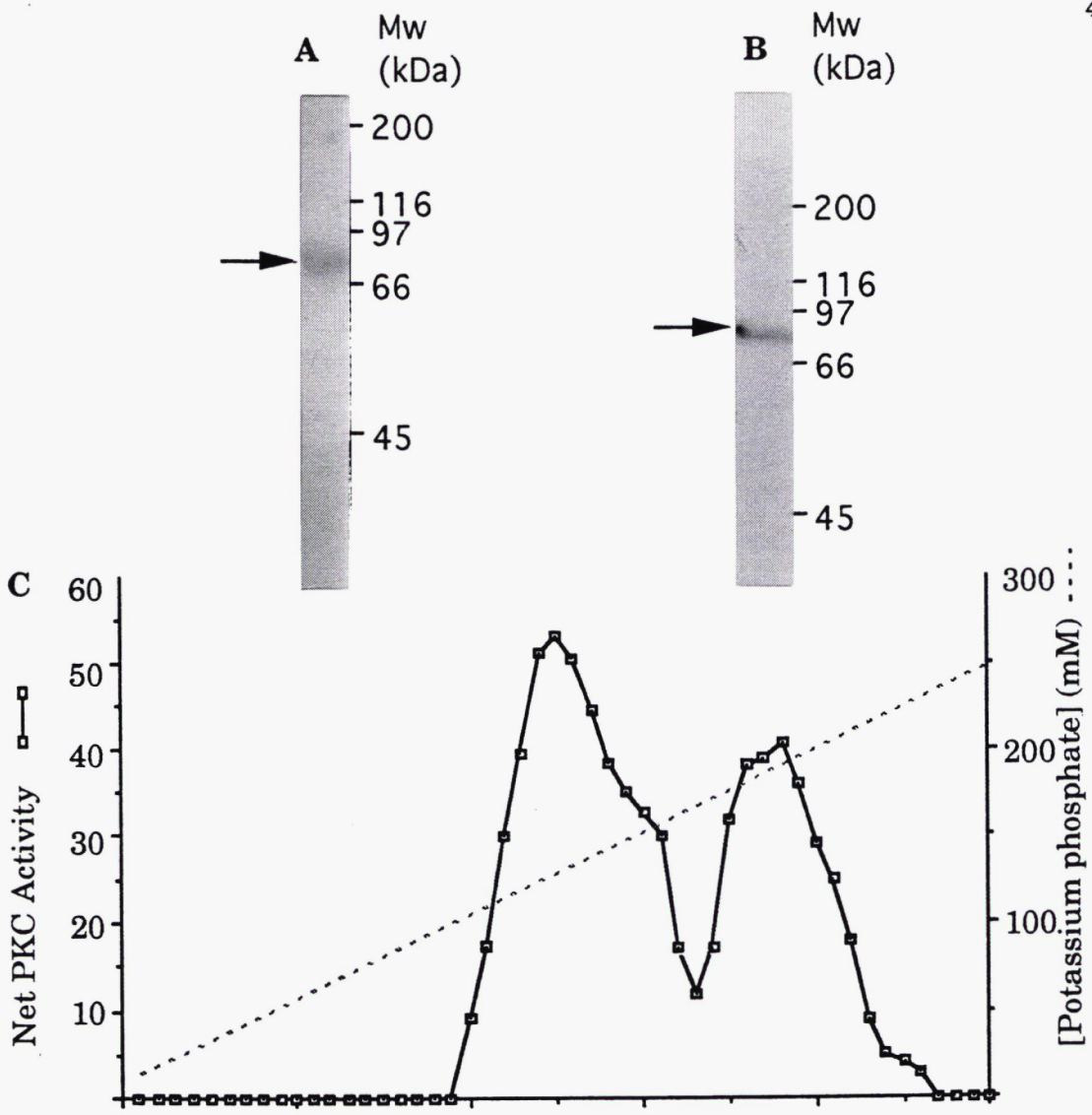

Figure 3.1 Identification of classical PKC isoenzymes in bovine parathyroid extracts using Western blotting and hydroxylapatite chromatography. (**A**) and (**B**) Chemiluminescent detection of PKC α (**A**) and PKC β (**B**) in whole parathyroid cell extracts. Arrows indicate immunospecefic bands. Approximate molecular weights are indicated. (**C**) Hydroxylapatite chromatography of DEAE-purified bovine parathyroid PKC. The calcium- and phospholipid- dependent PKC activity and approximate potassium phosphate concentration of each fraction are indicated.

coincided with the elution position of PKC α from bovine brain and displayed PKC α immunoreactivity. No PKC activity was found to elute below 100 mM potassium phosphate, the characteristic elution position for PKC γ.

3.1.2 Novel PKC isoenzymes

Isoenzyme-specific antisera failed to detect PKC δ, η, or θ immunoreactivity in bovine parathyroid cell fractions (Figures 3.2A, 3.2B and 3.2C). To ensure that the Western blotting conditions were sufficient for the detection of these isoenzymes, similar amounts of fractions derived from control tissues, which have been reported to contain the isoenzyme in question, were blotted in parallel with the parathyroid fractions.

Antibodies to PKC δ detected three bands with approximate molecular weights of 50, 74 and 76 kDa in the particulate fraction of bovine brain, whereas no bands were detected under these conditions in any of the bovine parathyroid fractions (Figure 3.2A). PKC δ, purified from brain tissue, displays two different molecular weights, dependent on its phosphorylation state (Ogita *et al*, 1992). Particulate fractions of brain tissue are particularly rich sources of this isoenzyme (Leibersperger *et al*, 1991). A variety of molecular weights between 74 and 79 kDa have been ascribed to PKC δ, including 74 and 76 kDa (Hug and Sarre, 1993). It is likely that PKC δ was detected by the blotting conditions in the present studies. The absence of detected bands in parathyroid fractions indicates the absence of this isoenzyme in the parathyroid cell.

Whereas no bands were detected in parathyroid extracts, two bands displaying PKC η immunoreactivity were observed in the triton-

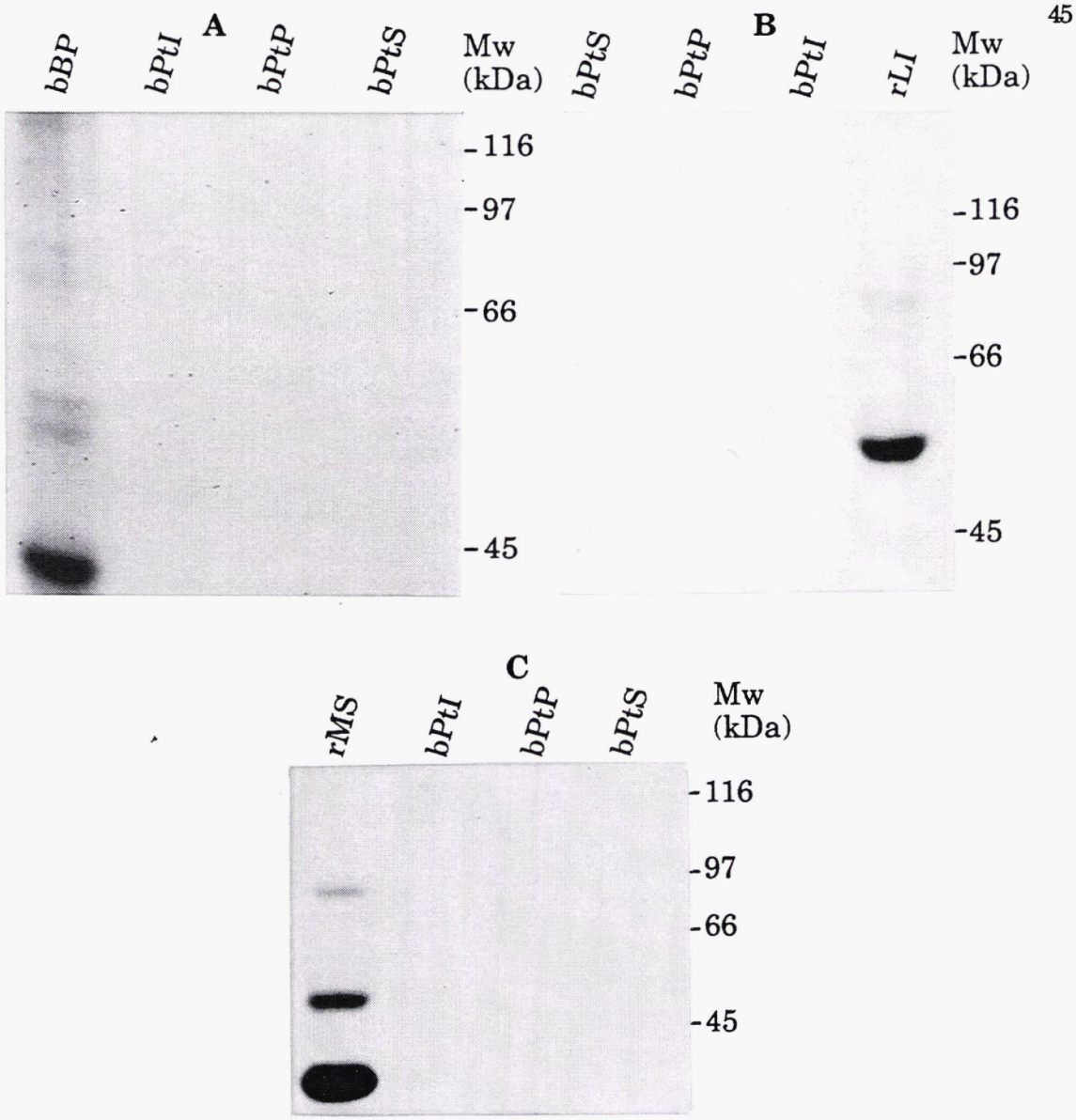

Figure 3.2 Western Blots of novel PKCs δ, η, and θ in bovine parathyroid and control fractions. Chemiluminescent images of blots of PKC δ (**A**), η (**B**) and θ (**C**) are shown. Approximately 20ug of bovine parathyroid insoluble (bPtI), particulate (bPtP) or soluble (bPtS) cell fractions were loaded per lane. Approximately 20 ug of either bovine brain particulate (bBP), rabbit lung insoluble (rLI) or rabbit biceps soluble (rMS) tissues were blotted in parallel as controls. Approximate molecular weights are indicated to the right of each image.

insoluble fraction of rabbit lung (Figure 3.2B). One band, displaying an approximate molecular weight of 82 kDa, corresponds to PKC η detected in murine and human lung tissue (Osada *et al*, 1990).

Three bands were detected by PKC θ-specific antisera in the soluble fraction of rabbit biceps, but not in any of the parathyroid fractions. Previous studies have described a 79 kDa PKC θ species in murine skeletal muscle extracts. The highest molecular weight band seen in Figure 3.2C corresponds closely with this molecular size.

Bands of appropriate molecular weight were identified in control tissues but not in parathyroid fractions, suggesting that PKC δ, η, and θ are not present, or are present but in very low abundance, in the parathyroid fractions. Therefore, subsequent studies to determine the immunospecificity of the bands detected by the antibodies for these isoenzymes were not performed.

Two proteins were detected by PKC ε-specific antisera in the soluble fraction of bovine parathyroid (Figure 3.3A). Only one of these bands, with an estimated molecular weight of 92 kDa, was found to be immunospecific, as determined by its disapearance when the blotting procedure was carried out using primary antibodies which had been preadsorbed with the antigenic peptide. Additionally, a band of similar molecular weight was detected in the soluble fraction of bovine brain tissue, a fraction known to be enriched in PKC ε (Koide *et al*, 1992).

3.1.3 Atypical PKC isoenzymes

Antiserum generated against the carboxyl-terminus of PKC ζ immunospecifically detected three proteins of differing molecular weights in soluble fractions of both brain and parathyroid (Figure

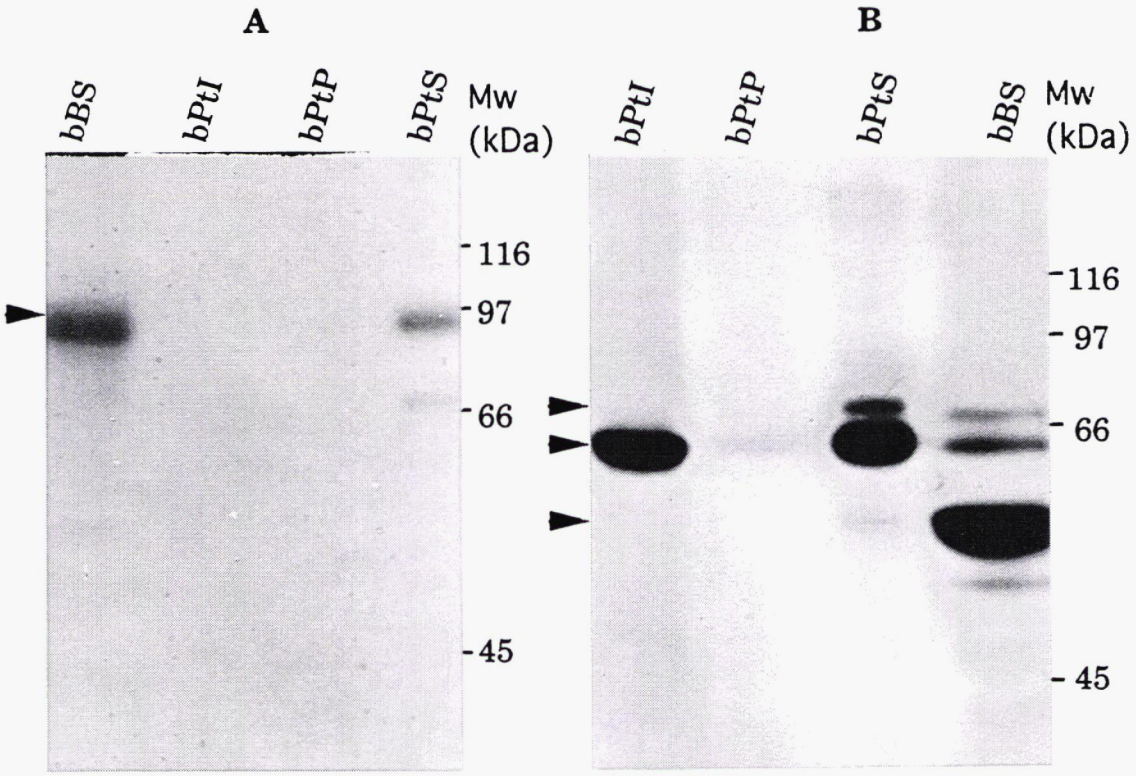

Figure 3.3 Western blots of novel PKC ε, and atypical PKC ζ in bovine parathyroid and control fractions. Chemiluminescent images of blots of PKC ε (**A**) and ζ (**B**) are shown. Approximately 20 ug of bovine parathyroid insoluble (bPtI), particulate (bPtP) or soluble (bPtS) cell fractions were loaded per lane. Approximately 20 ug of bovine brain soluble (bBS) tissue fraction was blotted in parallel as a control. Arrowheads indicate immunospecific bands. Approximate molecular weights are indicated to the right of each image.

3.3B). The highest molecular weight band, which was detected only in the soluble fraction of bovine parathyroid cells, had an approximate molecular weight of 72 kDa. This band probably corresponds to PKC ζ. A variety of molecular weights between 72 and 80 kDa have been ascribed to PKC ζ (reviewed by Zhou *et al*, 1994).

It has been demonstrated that PKC λ cross-reacts with antisera directed against the carboxyl-terminus of PKC ζ, and this atypical PKC isoenzyme is also present in brain tissue (Selbie *et al*, 1993). Thus Figure 3.3B also represents a blot for the detection of PKC λ in the parathyroid. A 64 kDa band detected in all bovine parathyroid fractions, as well as the brain control fraction, corresponds well with the molecular weight range used to describe PKC λ (Selbie *et al*, 1993, Zhou *et al*, 1994). A third band, appearing at approximately 55 kDa in both bovine brain and parathyroid soluble fractions was also found to be immunospecific, although it has not been described in the literature available.

Blots were not performed to determine the presence of PKC μ in parathyroid fractions as antibodies to this isoenzyme are not currently available.

3.2 Localization of classical PKCs and the secretory responses in parathyroid tissue to calcium and phorbol ester

The secretory responses of parathyroid tissue to high and low calcium in the presence or absence of PMA observed in this study were qualitatively similar to those observed in cultured bovine parathyroid cells (Tanguay *et al*, 1991). Intact PTH secretion was greatest for the low calcium treatment, followed by the high calcium treatment in the presence of PMA (see Figure 3.4). PMA treatment at low calcium resulted in slightly lower

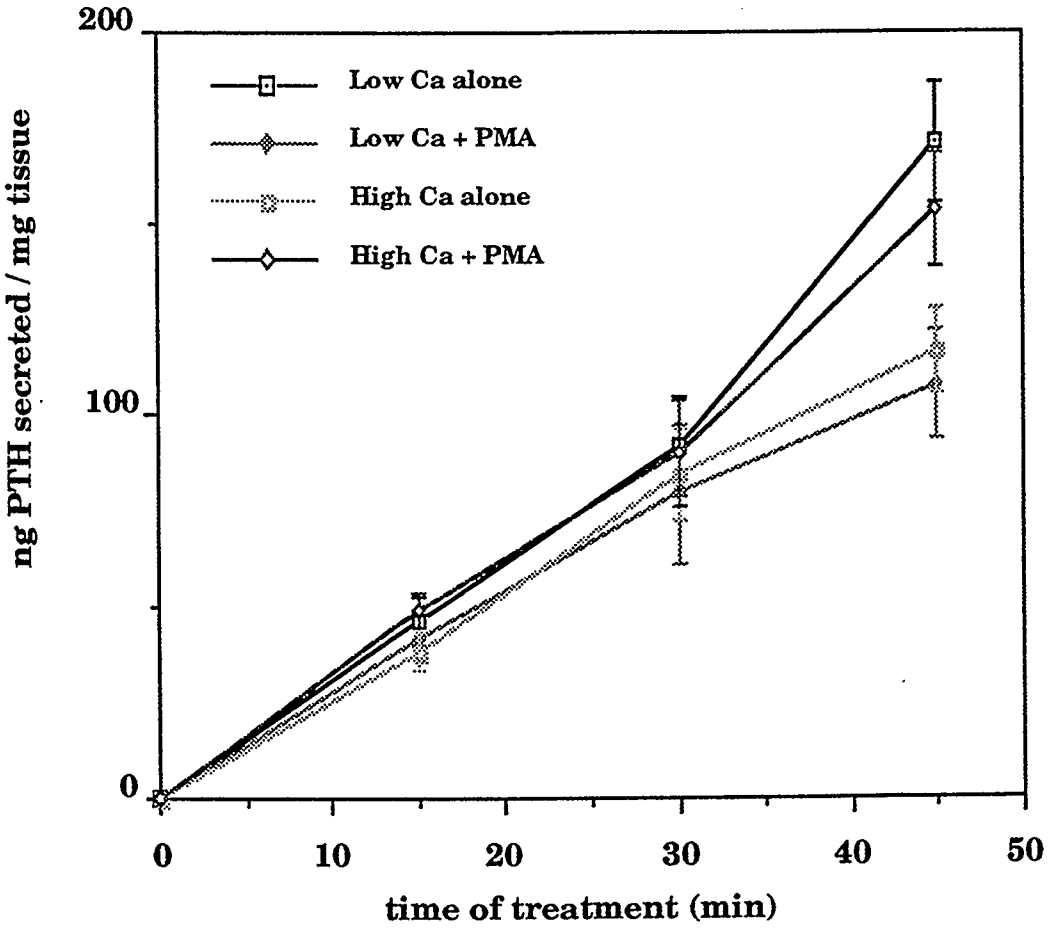

Figure 3.4 Time course of PTH secretion by bovine parathyroid tissue in response to high and low calcium in the absence or presence of PMA. PTH secretion, as measured by RIA with a detection preference for intact PTH, is shown as a function of time of treatment in 0.5 mM (Low Ca) or 2.0 mM (High Ca) calcium in the presence (+ PMA) or absence (alone) of 1.6 uM PMA. All RIA determinations were performed in triplicate. Data represents combined results of two separate experiments, bars indicate standard deviations.

secretory activity than high calcium alone, but both were much lower than the other two treatments. As can be seen in Figure 3.4, the most significant difference in secretion was observed between 30 and 45 minutes of treatment.

Although the relative amounts of PKC α and β in the soluble fractions did not significantly vary between the 45 minute treatments, membrane association of these isoenzymes did. In three separate experiments PKC α was found in the particulate fraction of only the low calcium treatment (Figure 3.5A). PKC β was detected in all particulate fractions, however it was most abundant under the condition of low calcium. The particulate fraction from the high calcium plus PMA treatment displayed the second greatest abundance of this isoenzyme (see Figure 3.5B). Trace amounts of PKC β could also be identified in the high calcium alone and low calcium plus PMA treatments. No immunospecific bands other than the 78 and 82 kDa bands shown in Figures 3.1A and 3.1B were detected by the α and β antisera, respectively, in these experiments.

3.3 Phosphorylation of bovine and human PTH by purified PKC

3.3.1 Phosphorylation of bovine PTH by PKC

Incorporation of radioactive phosphorus from γ^{32}P-ATP into bovine PTH (bPTH) initially increased as a function of time of incubation with PKC purified from bovine brain (in the presence of phospholipid, diolein and calcium). The phosphate incorporation into PTH increased to a plateau of phosphate to bPTH ratio of 1 to 3 (see Figure 3.6). This maximal value of phosphate incorporation was observed with PKC purified from bovine brain in two separate purifications, as well as from bovine parathyroid, although the rates of incorporation varied with the PKC preparation. A greater ratio

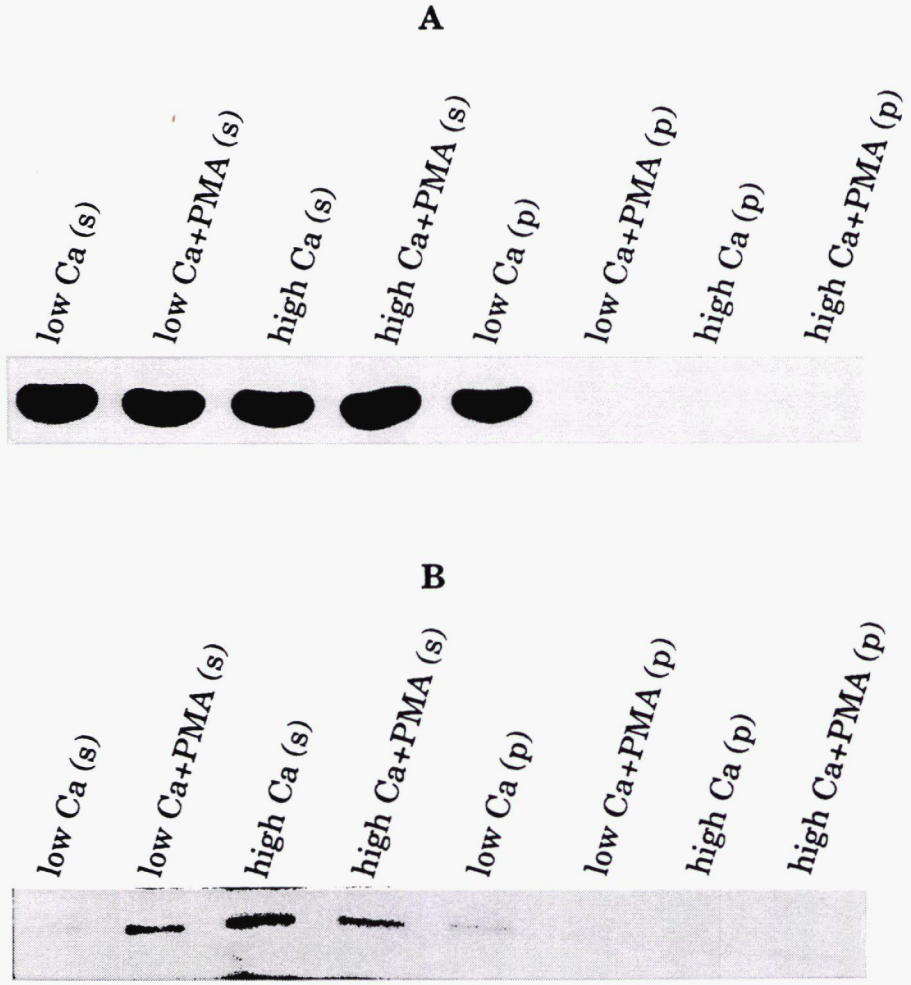

Figure 3.5 Western blots of PKC α and β in soluble and particulate fractions of treated parathyroid tissue. Chemiluminescent images of blots of PKC α (**A**) and PKC β (**B**) in soluble (s) and particulate (p) fractions are shown. Bovine parathyroid tissue was treated with high (2mM) or low (0.5mM) calcium in the presence or absence of 1.6uM PMA for 45 minutes. The tissue samples were then separated into soluble and particulate fractions. Fractions were loaded at 20 ug per lane and Western blotted for PKC α and β isoenzymes.

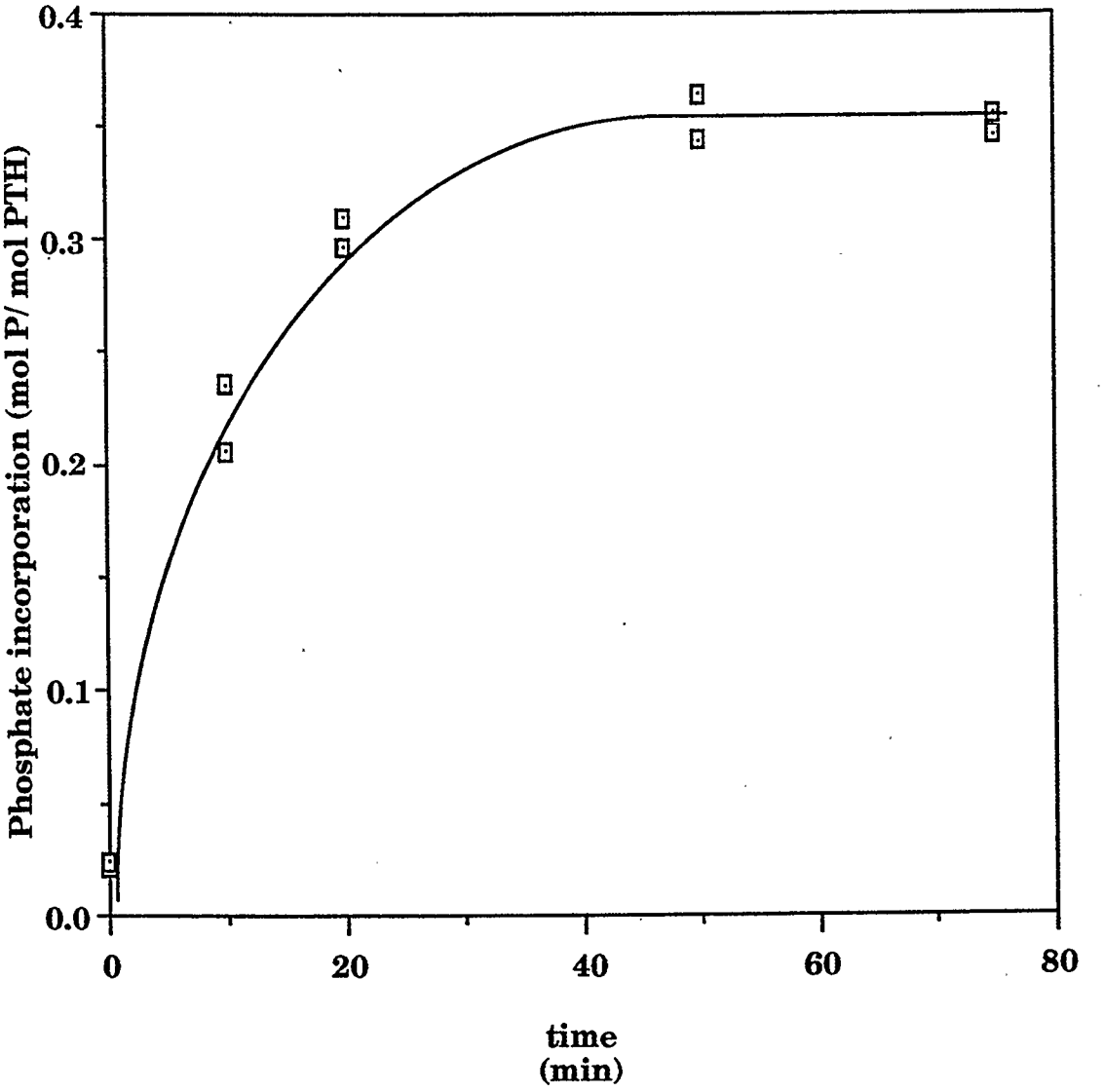

Figure 3.6 Time course of bovine PTH phosphorylation by purified bovine brain protein kinase C. Phosphate incorporation, calculated by scintillation counting of radioactive phosphate contained in PTH gel slices, is shown as a function of time of reaction.

of phosphate incorporation (up to one P to 1.5 bPTH) was calculated when reverse phase HPLC was used instead of gel electrophoresis to purify thephosphorylated PTH. The reason for this discrepancy may be due to losses of PTH in the reaction and purification procedure. These were accounted for in the HPLC procedure by RIA quantitation of PTH, but could not be accounted for in the gel purification procedure. Differences in radiation quenching due to sample content (gel slice versus HPLC eluant), may have also contributed to the discrepancy between the calculated phosphate incorporation by the two methods.

On reverse phase HPLC, PKC-phosphorylated bovine PTH (P-bPTH) typically eluted at the same position as untreated PTH (Figure 3.7). Phospho-amino acid analysis of acid hydrolysed P-bPTH revealed that most of the incorporated phosphorus co-migrated phosphoserine (see Figure 3.8). Phosphorylation of threonyl and tyrosil residues was not observed.

Extended (18 h) digestion of P-bPTH with Staphylococcus aureus V8 protease (V8 protease) produced two major phosphate containing peptides (Figure 3.9, peaks I and II); in addition to a peak of phosphorus radioactivity which eluted at the void volume of the column (see Figure 3.9). Amino-terminal sequence analysis of the peptide in peak II revealed that this peptide had an amino-terminus corresponding to position 39 of intact bPTH (refer to Figure 1.1). However, on several attempts, sequencing could not be performed beyond position 45 of intact bPTH, and no significant release of radioactivity from the sequencing support occurred in the six sequencing cycles.

Extended V8 protease digestion of the peptide contained in peak II resulted in the appearance of radioactivity that eluted at the position of peak

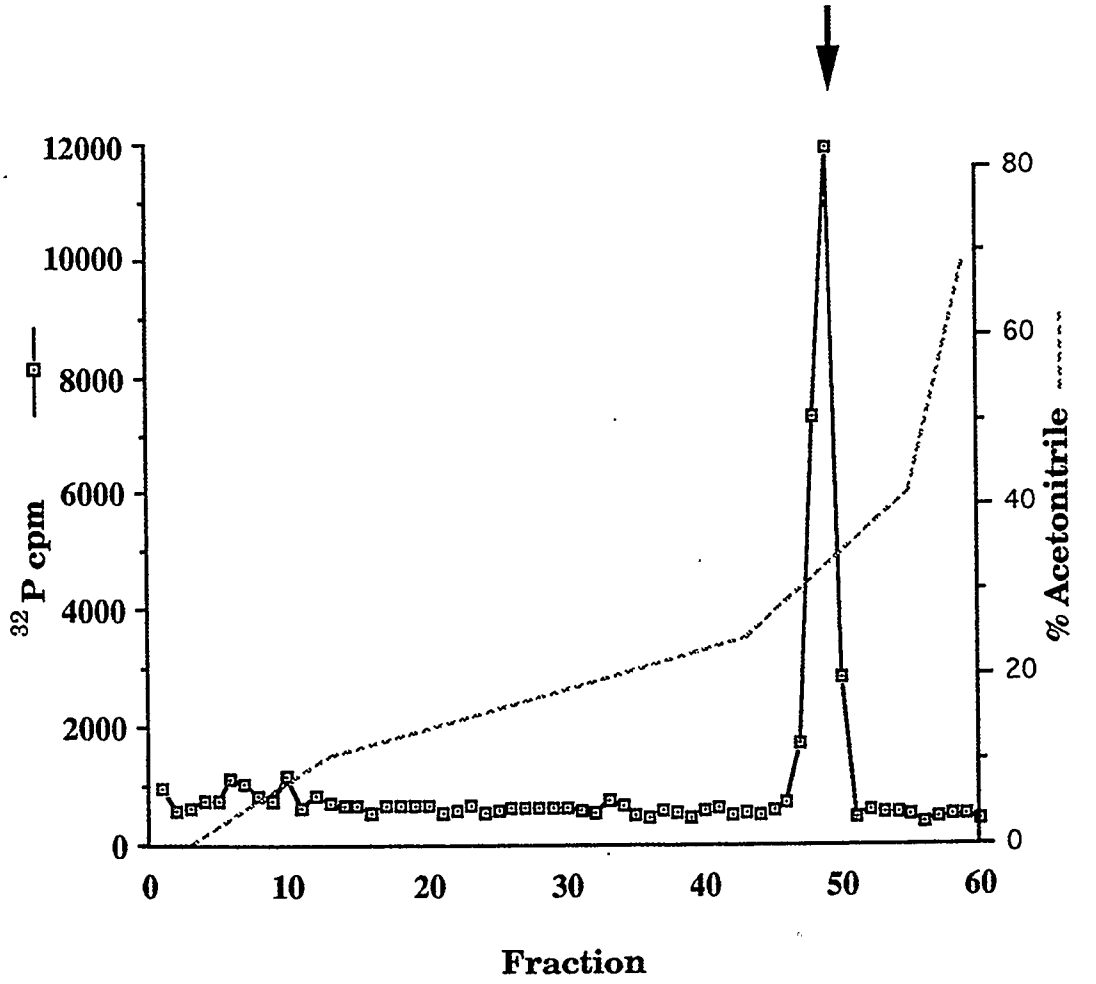

Figure 3.7 Reverse phase HPLC elution profile of PKC-phosphorylated bovine PTH. Radioactivity and percent acetonitrile of eluted fractions are shown. The arrow indicates the elution position of untreated bPTH, as well as position of maximal PTH immunoreactivity.

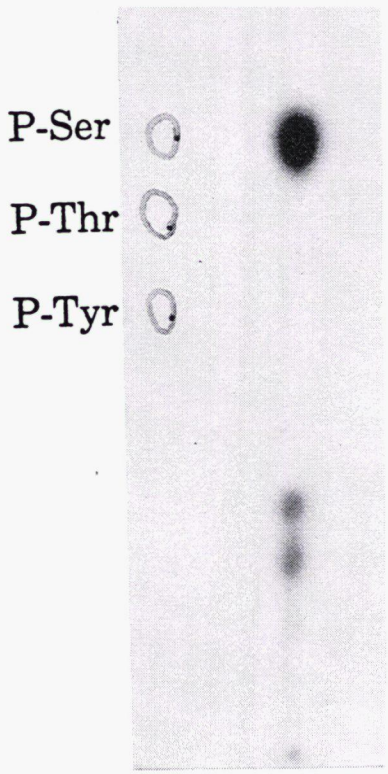

Figure 3.8 Phospho-amino acid analysis of PKC- phosphorylated bovine PTH. An autoradiogram of a thin layer electrophoresis plate on which hydrolysed PKC-phosphorylated bPTH was separated is shown. Migration positions of standard phospho-amino acids electrophoresed in parallel are shown on left side of autoradiogram.

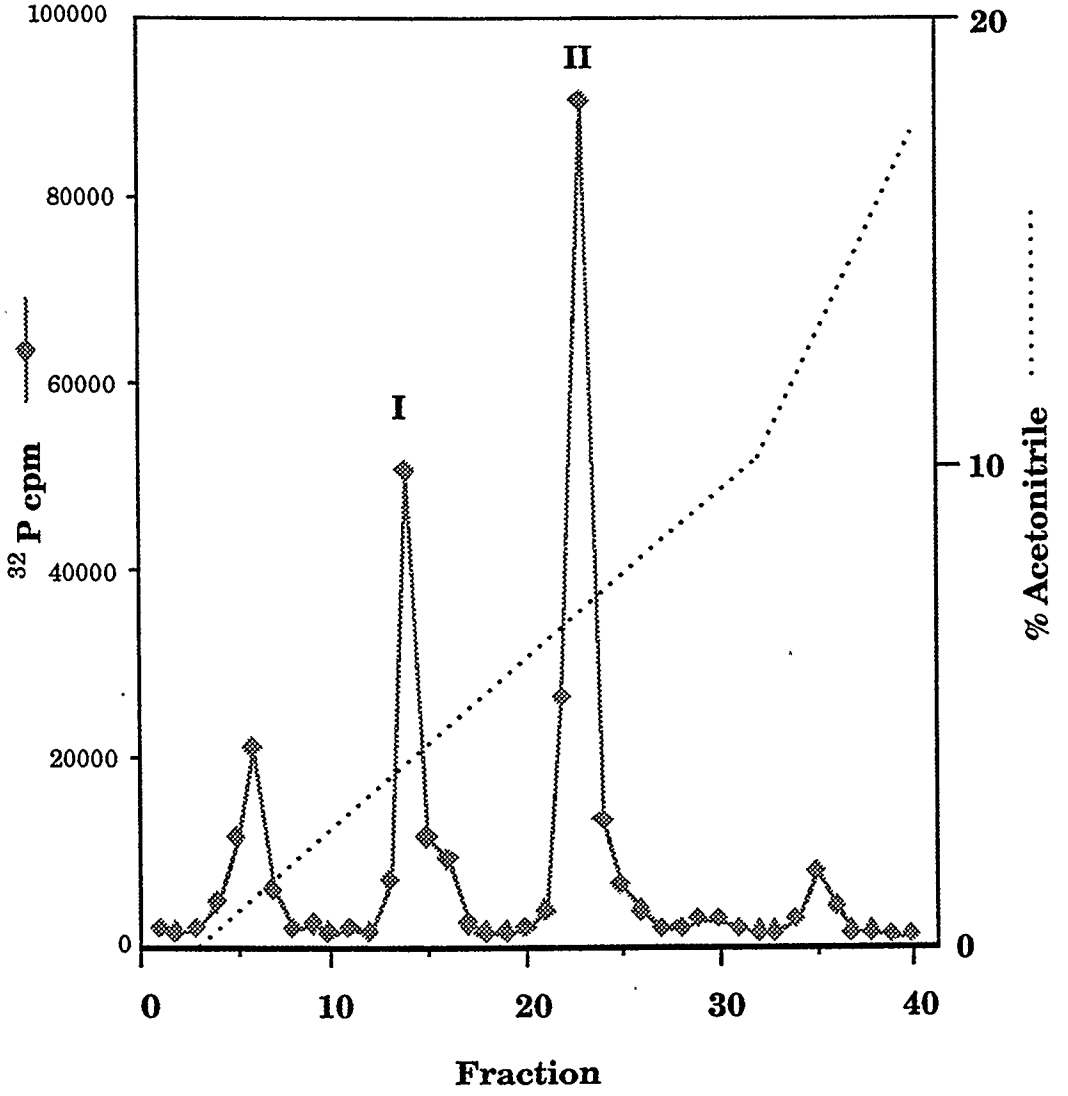

Figure 3.9 Reverse phase HPLC elution profile of *Staphylococcus aureus* V8 proteasedigest of PKC-phosphorylated bovine PTH. Radioactivity and percent acetonitrile of eluted fractions are shown. Fractions comprising peaks I and II were sent for sequence analysis.

I, with a corresponding decrease in radioacivity eluting in peak II. The results of amino-terminal sequence analysis of peptide contained in peak I are presented in Figure 3.10. The amino terminal position of this peptide was found to correspond with position 43 in intact bPTH (refer to Figure 1.1). The majority of the radioactivity was liberated in the sixth sequencing cycle, corresponding to serine 48 in intact bPTH. The seventh sequencing cycle revealed glutamine, the next amino acid in the sequence of bPTH. This indicated that the liberation of radioactivity in the previous sequencing step was due to the cleavage of a single amino acid from the peptide rather than the cleavage of the whole peptide from the sequencing support .

3.3.2 Phosphorylation of human PTH by PKC

PKC-phosphorylated human PTH (P-hPTH) eluted from the reverse phase HPLC column at a slightly lower percentage of acetonitrile than untreated PTH (Figure 3.11). Digestion of P-hPTH with V8 protease for 8 hours resulted in the production of a single ^{32}P-containing peptide, as determined by reverse phase HPLC separation of the digest (Figure 3.12). Amino-terminal sequencing of this peptide revealed that its amino terminus corresponded to residue 39 of intact hPTH (compare Figures 3.13 and 1.2). The majority of radioactivity was liberated at sequencing cycle 10, corresponding to serine 48 of intact hPTH. Two further sequencing cycles were performed and indicated that the liberation of radioactivity in cycle 10 was due to specific cleavage of the amino acid at this position.

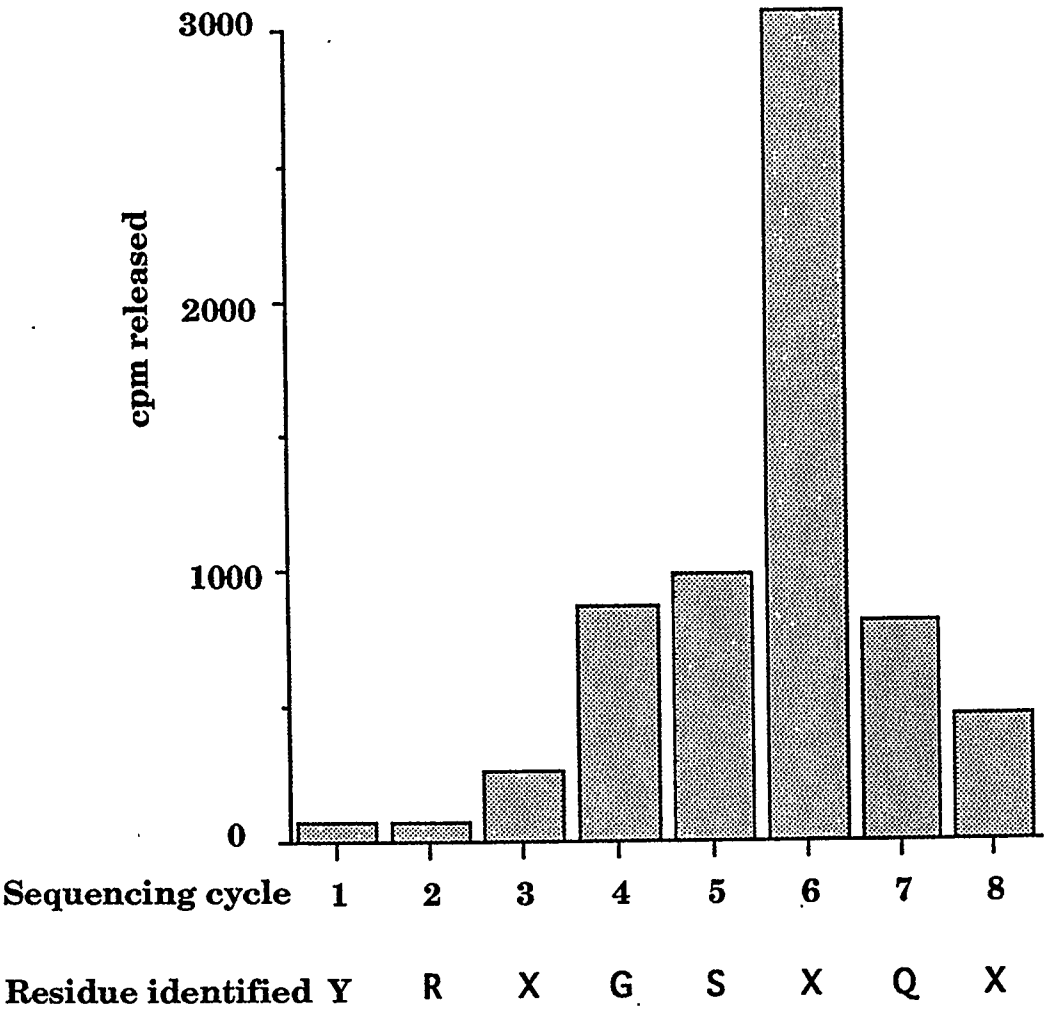

Figure 3.10 Amino-terminal sequence analysis of peak I peptide from *Staphylococcus aureus* V8 protease digest of PKC-phosphorylated bovine PTH. Radioactivity in addition to the identity of the amino acid released in each sequencing cycle is shown. One letter codes are used to designate amino acids, 'X' indicates that no amino acid could be positively identified.

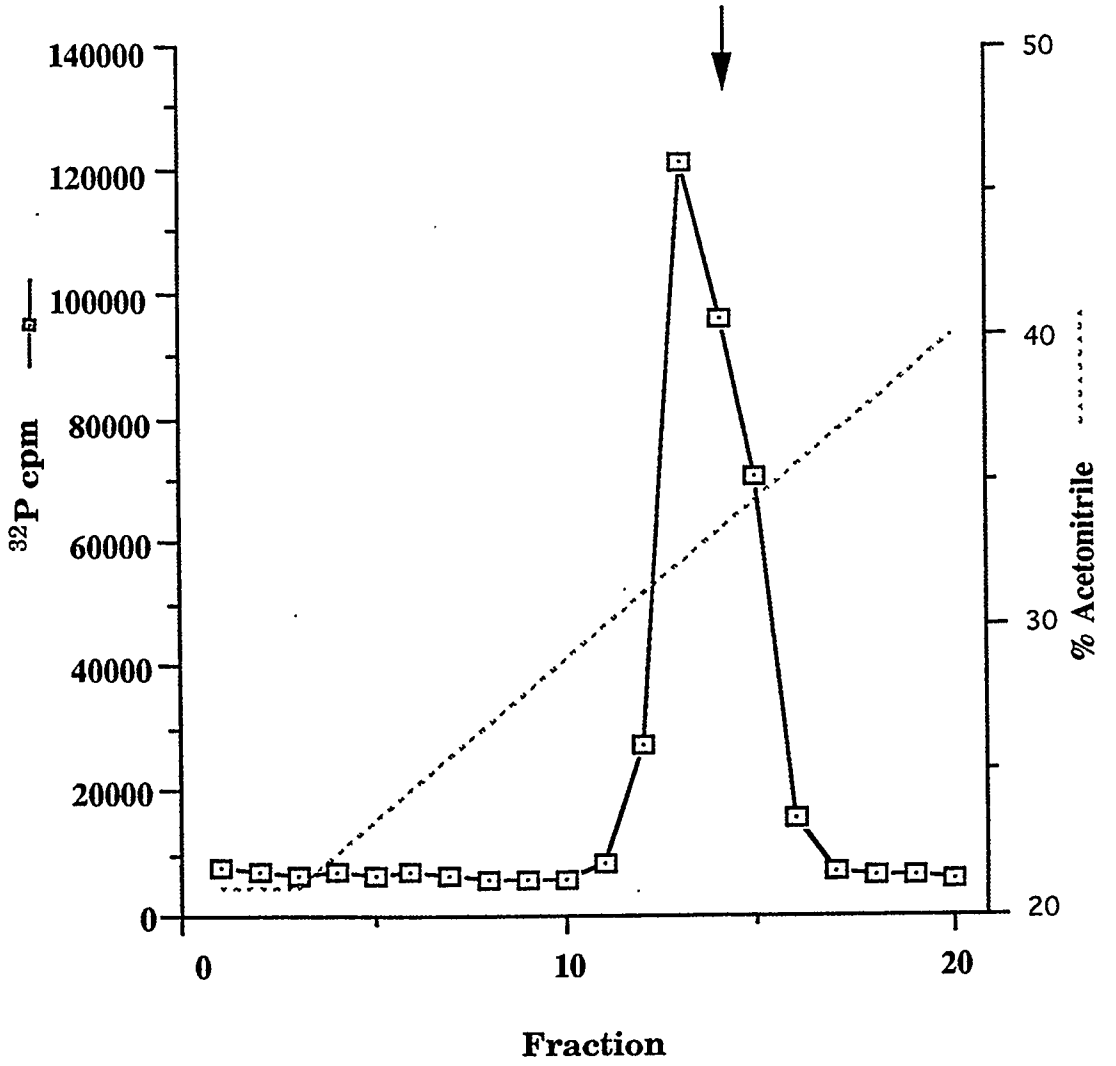

Figure 3.11 Reverse phase HPLC elution profile of PKC-phosphorylated human PTH. Radioactivity and percent acetonitrile of eluted fractions are shown. The arrow indicates the elution position of untreated hPTH.

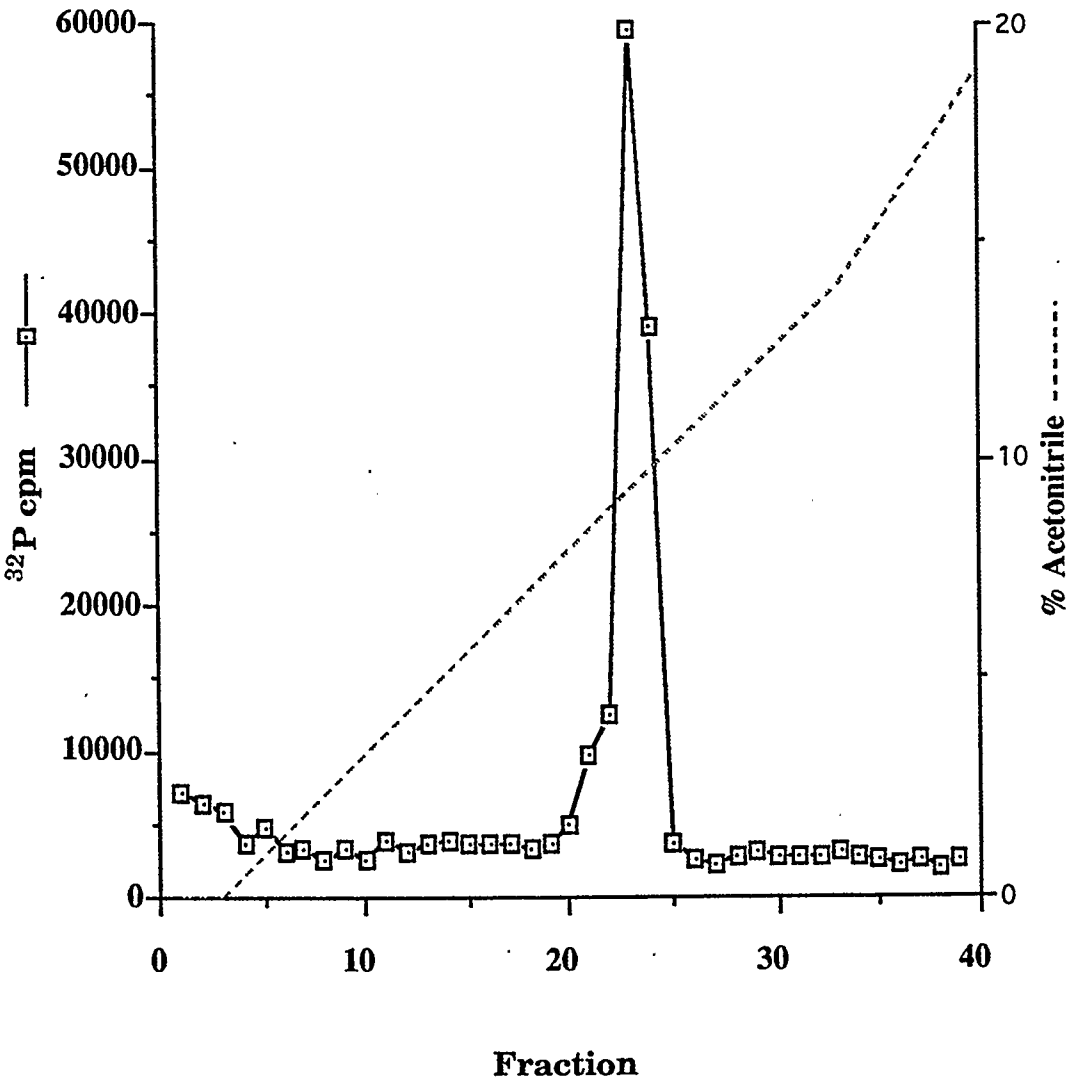

Figure 3.12 Reverse phase HPLC elution profile of *Staphylococcus aureus* V8 protease digest of PKC-phosphorylated human PTH. Radioactivity and percent acetonitrile of eluted fractions are shown. The fractions comprising the single radioactive peak were pooled and sent for sequence analysis.

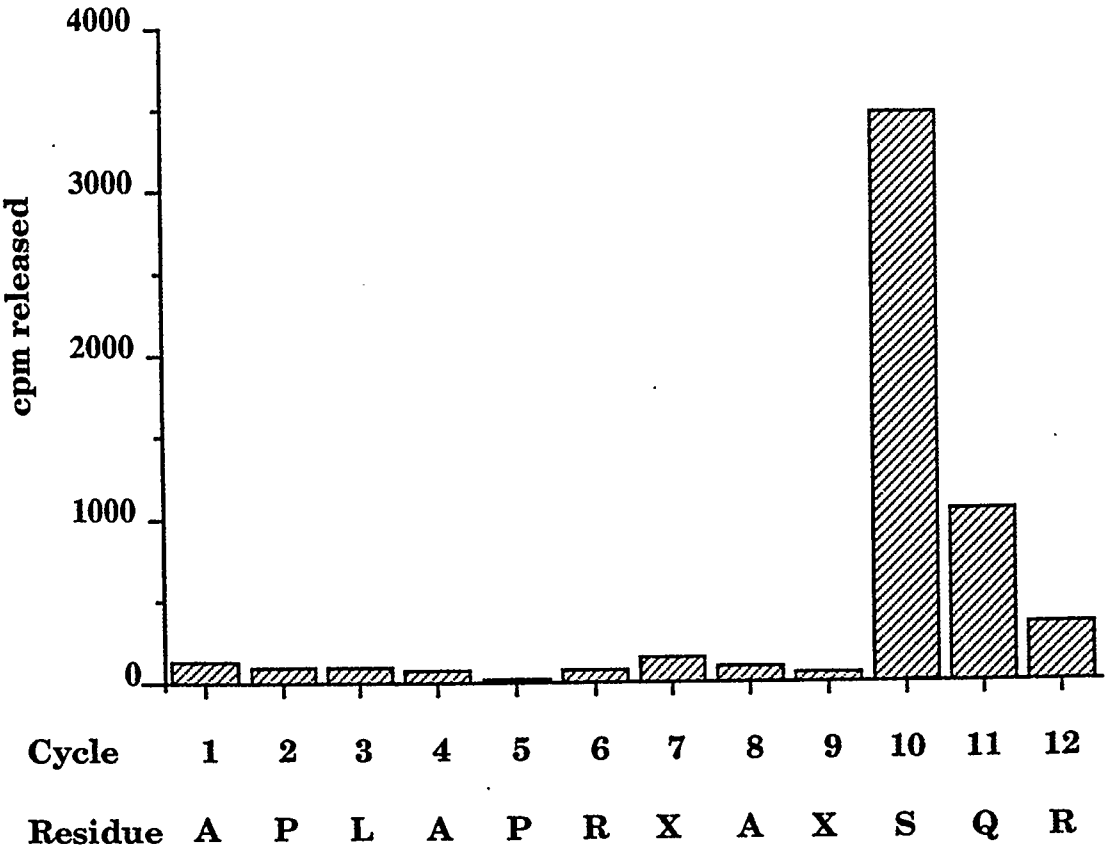

Figure 3.13 Amino-terminal sequence analysis of phosphopeptide from *Staphylococcus aureus* V8 protease digest of PKC-phosphorylated human PTH. Radioactivity in addition to the identity of the amino acid released in each sequencing cycle is shown. One letter codes are used to designate amino acids, 'X' indicates that no amino acid could be positively identified.

Discussion

4.1 Identification of protein kinase C isoenzymes in the bovine parathyroid

4.1.1 Classical PKC isoenzymes

Two classical PKC isoenzymes were identified in bovine parathyroid extracts using immunological and chromatographic methods. Western blot analysis revealed that parathyroid PKC α has a molecular weight of 78 kDa and PKC β has a molecular weight of 82 kDa. Hydroxylapatite chromatography of partially purified bovine parathyroid PKC revealed two peaks of classical PKC activity. These peaks corresponded to the identified elution positions of PKC β and PKC α. Classical PKC activity was not found in the fractions at which PKC γ typically elutes on hydroxylapatite chromatography.

Currently, PKC γ has been identified only in brain tissue. The results of the present study indicate that this isoenzyme is not expressed in the parathyroid, and are consistent with this tissue specificity of PKC γ (reviewed by Hug and Sarre, 1993, Dekker and Parker, 1994). PKC α is described as a relatively ubiquitous isoenzyme, and the present study indicates this isoenzyme is also present in the parathyroid. Either or both of the two splice variants of PKC β have been identified in a wide range of tissues, including several secretory tissues (reviewed by Kikkawa *et al*, 1987). Although the current study did not determine the specific splice variant composition, it clearly indicated that PKC β is present in the parathyroid.

The identification of two calcium- and DAG-responsive PKC isoenzymes in the parathyroid suggests that these play a role in

parathyroid cell regulation. The possible involvement of these cPKCs in the parathyroid secretory response will be discussed in section 4.2.

4.1.2 Novel PKC isoenzymes

Only one of the four novel PKC isoenzymes were detected in bovine parathyroid cell fractions. Proteins of the appropriate molecular weight were detected in control tissue fractions by antisera to PKC δ, η and θ, but not in parathyroid fractions. Therefore, it is unlikely that these isoenzymes are present at detectable levels in parathyroid cells under normal conditions. PKC ε was immunospecifically detected in bovine parathyroid soluble fractions. This isoenzyme exhibited an apparent molecular weight of approximately 92 kDa when extracted from both parathyroid and brain tissues.

PKC δ is present in a relatively wide range of tissues, although there are a few tissue which have been found to lack this isoenzyme (reviewed by Hug and Sarre, 1993). The studies presented here suggest that the parathyroid does not express the PKC δ isoenzyme under normal conditions, and thus is not necessary for short term responses in the parathyroid. PKC η and θ isoenzymes display a relatively narrow tissue distribution. The η isoenzyme is found predominantley in epithelial tissue while the θ isoenzyme is present primarily in skeletal muscle (Bacher *et al*, 1991, Osada *et al*, 1990, Osada *et al*, 1992). These isoenzymes were not identified in the parathyroid cell extracts in the present study. PKC ε is a component of the soluble fraction in a variety of unstimulated cells. Stimulation of these cells by certain agents results in membrane association of this isoenzyme, presumably indicating its activation (Hug and Sarre, 1993). The identification of PKC ε in the soluble fraction of

parathyroid cells harvested from normocalcemic (1.25 mM Ca_e) conditions suggests that this isoenzyme is not significantly activated under these conditions.

The novel PKC isoenzymes are activated by DAG and phorbol esters, but not by calcium. Identification of a novel PKC isoenzyme in the parathyroid suggests that it may play a role in the mediation of certain aspects of cellular functions within this gland. Of particular interest is the possibility that novel PKCs play a role in the degradation of PTH in the parathyroid. Although other PKC isoenzymes may affect the degradation of PTH, the involvement of a novel PKC isoenzyme in this process is suggested by studies using PKC inhibitors to reverse the effects of phorbol esters on secretion and degradation. In one such study it was found that, although the PKC inhibitor H-7 was able to reverse the effects of PMA on the secretion of PTH, it was unable to completely reverse the effects of PMA on the degradation of PTH. In studies using endogenous substrates novel PKC isoenzymes were found to be much less sensitive to inhibitors like H-7 than the classical PKCs (Koide, 1992, Oudinet *et al*,199 ,Wilkinson and Hallam, 1994). Thus it is possible that the PMA-activated, H-7-resistant degradation of PTH is mediated by a novel PKC isoenzyme. Furthermore, arachidonic acid (AA) is a potent activator of nPKC ε (Koide *et al*, 1992), and the addition of AA to parathyroid cells at low calcium causes a decrease in the total amount of intact PTH secreted (Bourdeau *et al*, 1992). Also, cellular AA levels increase when the parathyroid cell is exposed to high calcium, a condition in which PTH degradation is enhanced (Bourdeau *et al*, 1992, Tanguay *et al*, 1991). These findings suggest a role of nPKC ε in the degradation of PTH.

4.1.3 Atypical PKC isoenzymes

Two atypical PKC isoenzymes, PKC λ and PKC ζ, were tentatively identified in soluble fractions of parathyroid and brain. PKC λ is highly homologous to PKC ζ, and is recognized by antibodies directed against the carboxyl terminus of PKC ζ, suggesting that the 64 kDa species detected by the PKC ζ antiserum is PKC λ (Selbie *et al*, 1993). PKC λ is expressed in brain tissue. Thus, it is likely that the 64 kDa bands detected in the brain soluble fraction and in the triton-insoluble, particulate and soluble fractions of the parathyroid in the present study represent PKC λ. However, a splice variant of PKC ζ mRNA has been identified in brain tissue suggesting the alternative possibiltiy that the 64 kDa bands identified in this study represent ζ', the translation product of this smaller mRNA (Zhou *et al*, 1994 and references therein). PKC ζ' has not been characterized independently of PKC ζ, but it may be expressed in brain tissues (reviewed by Zhou *et al*, 1994). Presently no isoenzyme-specific antisera are available for PKC μ, and thus it remains unknown as to whether this isoenzyme is present in the parathyroid.

Both PKC ζ and λ are found in a variety of tissues (reviewed by Zhou *et al*, 1994). Although in some cells, agonist treatment results in the translocation of PKC ζ to the membrane fraction, in other cell types PKC ζ is associated with the particulate fraction in the resting state (Zhou *et al*, 1994). It is unclear whether this isoenzyme requires membrane association for activation. The identification of PKC ζ in only the soluble extracts of bovine parathyroid cells harvested from normocalcemic media may or may not indicate that it is inactive under these conditions. There is some evidence that PKC ζ is activated by unsaturated fatty acids, such as

arachidonic acid, and products of PI-3' kinase (Reviewed by Hug and Sarre, 1993 and Parker and Dekker, 1994, Nakanashi and Exton, 1992). These potential activators accumulate in parathyroid cells exposed to high extracellular calcium (Hawkins *et al*, 1989, Bourdeau *et al*, 1992), suggesting the possibility that PKC ζ may be activated under these conditions. PKC λ is expressed in a variety of cell types, including secretory cells (Selbie *et al*, 1993). The tentative identification of PKC λ in parathyroid fractions is consistent with the wide tissue distribution of this isoenzyme. At present, little is known about the activational characteristics of PKC λ, although phosphatidylserine and free fatty acids are thought to activate this isoenzyme (Akimoto *et al*, 1994, reviewed by Nishizuka *et al*, 1992). Identification of the role of PKC λ in the parathyroid awaits further characterization of this isoenzyme. The presence of PKC λ in all three parathyroid fractions examined suggests two possibilities: It is possible that this isoenzyme performs a variety of functions in the parathyroid cell, and thus is present in all fractions. Alternatively, it is possible that PKC λ is activated under the normocalcemic conditions that the parathyroid cells were harvested from. The finding that a greater amount of 64 kDa PKC ζ immunoreactivity is present in the soluble parathyroid fraction than other fractions suggests that this may be the primary site of PKC λ localization in the parathyroid when not activated. Activation may then involve association with both the membrane and cytoskeletal elements, which are components of the particulate and triton insoluble fractions, respectively.

PKC ζ and PKC λ are thought to mediate a host of cellular activities, including regulation of gene expression, cell differentiation, phagocytosis and secretion (reviewed by Zhou *et al*, 1994). At present however, the

understanding of the regulatory features of these atypical isoenzymes is minimal. Discrepancies regarding the membrane association in the resting state, possible secondary effects of PMA on activation, combined with lack of knowledge about endogenous activators and substrates make the role of these isoenzymes in the parathyroid difficult to predict.

4.1.4 Further studies on specific isoenzymes

The identification of five different PKC polypeptides in parathyroid extracts, each reported to display different activational characteristics and substrate preference, strongly suggests that these isoenzymes may perform different roles in this tissue type. Several studies will clarify the roles of these isoenzymes in the parathyroid. These include: 1) identification of endogenous substrates of each isoenzyme; 2) examination of intracellular localization of each isoenzyme in response to various treatments; and 3) identification of the specific PKC activators produced in the parathyroid in response to these treatments.

4.2 The involvement of PKCs in PTH secretion

4.2.1 PTH secretion and cPKC membrane association

Membrane association of PKC has been found to precede or coincide with increased secretion in a variety of cell types (Reviewed by Nishizuka, 1992, Dekker and Parker, 1994, Hug and Sarre, 1993). The membrane association of the PKC isoenzymes in this study was assessed 45 minutes after initiation of treatment. This time was chosen because of the nature of the PTH secretory response observed in parathyroid tissue preparations. In the intact parathyroid, where intercellular communications and circulation are undisturbed, the secretory response to changes in extracellular calcium is almost instantaneous (Brown, 1991, and

references therein). Previous studies in which membrane association of classical PKC activity was measured were performed using dispersed parathyroid cell preparations. In these studies, secretory responses to extracellular calcium concentrations occurred within minutes of treatment. Changes in the membrane association of cPKC activity were also observed in this time span (Kobayashi *et al*, 1988; Morrissey, 1988). The secretory response of the parathyroid tissue preparations used in this study occurred significantly only between 30 and 45 minutes of treatment. Thus, it was assumed that the parathyroid tissue preparations were able to perceive differences in the composition of the media after approximately 30 minutes of treatment, possibly due to the increased time required for diffusion into the cells within the tissue. The differences in membrane association of PKC isoenzymes in this study are therefore thought to be indicative of alterations in the secretory response of the parathyroid.

After 45 minutes of treatment, membrane association of PKC in other systems is thought to be indicative of transcriptional changes (Nishizuka, 1992). In the parathyroid increases in PTH mRNA synthesis are not observed within one hour of alteration of Ca_e, or phorbol ester treatment (Hawa *et al*, 1993, Clarke *et al*, 1993). Furthermore, PTH is constitutively synthesized at a near maximal rate in the parathyroid, and there is little evidence to indicate that the level of PTH synthesis is altered by short term (<1H) manipulation of Ca_e or phorbol ester treatment of bovine parathyroid cells (Watson and Hanley, 1992). Therefore it is reasonable to postulate that the treatments performed in the current study alter the level of secretion by effects on transport and release and/or degradative processes.

In experiments using dispersed cells, several researchers have found increased membrane association of cPKC activity in response to low calcium treatment (Kobayashi *et al*, 1988; Morrissey, 1988) and high calcium plus PMA treatment (Kobayashi *et al*, 1988, Membreno *et al*, 1989). In the present study, localization of the specific cPKC species in response to high and low calcium in the presence or absence of PMA was examined. It was found that PKC α associates with the membrane only during low calcium-stimulated secretion. PKC β, although present in the membrane fractions after all treatments, was found to a greater extent in the membrane fraction under both conditions of increased PTH secretion (low calcium and high calcium plus PMA). Although there has been some debate as to whether association of PKC isoenzymes with particulate fractions indicates activation (Nishizuka, 1992), it is evident from these studies that membrane association of cPKC isoenzymes coincides with states of increased secretion in the parathyroid.

The present study supports the suggestion that PKC membrane association is involved in the parathyroid secretory process. However, the precise molecular nature of cPKC activation in this gland remains elusive. Phorbol ester treatment blunts the accumulation of intracellular calcium and inositol phosphates, and presumably DAG, in response to high Ca_e, resulting in similar intracellular conditions to those seen at low Ca_e (Racke and Nemeth 1993a; Membreno *et al*, 1989; Kifor and Brown, 1988). In this study, as well as other studies (Morrissey, 1988, Membreno *et al*, 1989, Kobayashi *et al*, 1988), increased cPKC membrane association is greatest under these conditions of seemingly low levels of classical PKC activators. One possible explanation for this finding is that at low Ca_e, the levels of PKC

activators are already high enough to promote membrane association of cPKCs. This is supported by early studies which found that when Ca_i was decreased to subphysiological levels by decreasing or chelating Ca_e, secretion decreased (Nygren *et al*, 1987).

At high Ca_e the cellular levels of calcium and DAG are elevated in comparison to those at low Ca_e. However, cPKC membrane association decreases, as does the level of PTH secretion (This study, Morrissey 1988, Kobayashi *et al*, 1988). It is possible that at high Ca_e, increased Ca_i causes the activation of the calpains, a group of calcium-activated proteases which are thought to be responsible for intracellular degradation of membrane-associated PKC (reviewed by Kikkawa *et al*, 1987). Both milli-calpain and micro-calpain have been identified in the parathyroid (Watson *et al*, 1995). Alternatively, the increased levels of DAG at high Ca_e may stimulate membrane lipid turnover (reviewed by Nishizuka, 1992). DAG-stimulated phosphatidylcholine hydrolysis leading to the production of alkylglycerol compounds has been found in other systems (Bishop *et al*, 1992). Alkylglycerols are endogenous inhibitors of PKC, and thus may cause decreased membrane association of PKC (Robinson *et al*, 1995). Other products of membrane lipid turnover, such as the sphingosines are also endogenous PKC inhibitors and may be produced by the parathyroid in response to high Ca_e. Treatment of parathyroid cells with sphingamine results in the inhibition of PTH secretion (Clarke *et al*, 1993). Therefore, it is possible that the increased DAG levels in response to elevated Ca_e actually secondarily inhibit the activation of PKC, resulting in the decreased membrane association of classical PKC isoenzymes observed in the present study. This negative-feedback control may be exerted by DAG-responsive

non-classical PKC isoenzymes, and therefore could be elicited at low calcium by PMA.

The differences in the membrane association of the two classical PKC isoenzymes found in this study indicate that PKC α and β are activated differentially in the parathyroid. As mentioned earlier, PMA treatment blunts the effects of high extracellular calcium on the intracellular accumulation of calcium and inositol phosphates. Therefore intracellular environment in the high calcium, PMA-treated parathyroid cell is similar to that of the cell at low Ca_e. This would may explain the similarities in the PKC β membrane association and secretory response under these two conditions. The finding that PKC α is present in the membrane fraction of only the low calcium-treated cell may be due to differences in other PKC activators, such as specific fatty acids, in the cell under these two conditions (Dekker and Parker, 1994). Several fatty acids are known to differentially effect the classical PKC isoenzymes (reviewed by Kikkawa *et al*, 1987).

The differential membrane association of PKC α and β also suggests that these isoenzymes may have distinct roles in the parathyroid response to extracellular calcium. A rough correlation between PKC β membrane association and PTH secretion can be made, suggesting that this isoenzyme may be responsible for the activation of the cellular secretion mechanisms. The phosphorylation of two proteins was enhanced when parathyroid cells were exposed to low calcium as well as high calcium plus PMA treatments (Morrissey, 1988). These proteins were found to be acidic, and had approximate molecular weights of 100 and 20 kDa. Since PKC β is activated under these conditions, it is possible that this isoenzyme phosphorylates

either or both of these proteins. In this regard, it is interesting to note that a 20 kDa protein which is important in activation of secretion has been identified in several specialized secretory cells (Wallent *et al*, 1992). Furthermore, the activating role of this 20 kDa protein is kinase inhibitor-sensitive and dependent upon the presence of Mg-ATP (Hay and Martin, 1992). It is possible that the 20 kDa protein found in parathyroid cells under conditions of increased PKC β membrane association is the 20 kDa secretory protein, suggesting the direct role of this PKC isoenzyme in PTH secretion.

Alternatively, it has been theorized that the role of PKC in the parathyroid is to phosphorylate the cell surface calcium receptor, thereby decreasing the sensitivity of the parathyroid to extracellular calcium (Brown *et al*, 1992, Racke and Nemeth, 1993b). This is supported by the decreased intracellular response of the parathyroid to elevated Ca_e upon phorbol ester treatment (Kifor *et al*, 1990, Racke and Nemeth 1993a). Further, parathyroid glands from humans that have mutations in a single PKC consensus sequence in the calcium receptor display decreased calcium sensitivity (Pollak *et al*, 1993). The calcium receptor has an estimated molecular weight of 120 kDa, and may correspond to the 100 kDa phosphoprotein identified in the early labeling experiments (Morrissey, 1988). However, the finding that the 100 kDa protein is phosphorylated at low calcium does not support the proposed link between phosphorylation of this protein and decreased calcium responsiveness, since low Ca_e pretreatment does not blunt the parathyroid response to subsequent exposure to elevated Ca_e (Racke and Nemeth, 1993a).

PKC α may contribute to the secretory process at low calcium but probably has other functions, as it is not present in the membranes of cells stimulated to secrete at high Ca_e by PMA. A low molecular weight acidic protein was phosphorylated at low Ca_e, but not at high Ca_e in the presence or absence of PMA (Morrissey, 1988). Since the low calcium treatment was the only one which promoted membrane association of PKC α in the present study, it is possible that this protein may be its endogenous substrate. Further characterization of this low molecular weight acidic phosphoprotein may allow elucidation of the specific molecular role of PKC α in the parathyroid.

4.2.2 Further studies on the involvement of PKCs in the secretion of PTH

This study adds to the multitude of reports which suggest a role for PKCs in the secretion of PTH. Further work to identify the specific PKC activators and inhibitors produced in the parathyroid may provide insights into the regulation of these isoenzymes in the parathyroid. Identification of the specific substrates of the cPKCs would allow greater understanding of the molecular basis of secretion in general. Finally, membrane association of the cPKCs in response to varoius times of treatment should be determined. By this means, the possibility that the membrane association observed in the present study is linked to later transcriptional changes could be examined.

4.3 Phosphorylation of PTH by protein kinase C

4.3.1 *In vitro* phosphorylation of PTH by PKC

The possibility that protein kinase C may directly phosphorylate PTH was investigated in this study. Protein kinase C purified from bovine brain as well as from bovine parathyroid were able to phosphorylate bovine PTH

in vitro. PKC purified from three separate preparations, two from bovine brain and one from bovine parathyroid, produced maximum phosphate incorporation of 1P : 3bPTH, when phosphate incorporation was determined by counting incorporation of radioactivity into gel slices containing bPTH. When phosphate incorporation was measured by HPLC analysis, a higher value was calculated (1:1.5 for one determination). This difference may be due to the differences in the determination of PTH in each method. Using the gel method, it was assumed that all of the PTH loaded onto the gel was contained in the gel slice excised from the gel. In the HPLC method, PTH content was determined by RIA. Losses due to proteolysis, adherence to reaction tubes, and transfer as well as losses due to staining and destaining of the gel prior to excising the PTH bands were not accounted for in the gel method, and may account for the differences in the phosphate incorporation observed by these two methods. Differences in radiation quenching by samples produced by the two methods may have also lead to discrepancies in the observed phosphate incorporation.

Because brain is an abundant source of protein kinase C, PKC purified from bovine brain (rather than from parathyroid tissue) was used for preparative phosphorylations of PTH. Phosphorylation did not significantly alter HPLC elution characteristics of either bPTH or hPTH. Amino-terminal sequence analysis of phospho-peptides produced by *Staphylococcus aureus* V8 protease digestion of PKC-phosphorylated PTH revealed that serine 48 is phosphorylated in both bovine and human PTH.

Serine 48 is a highly conserved residue in the PTH sequence of several species (reviewed by Cohn and MacGregor, 1981). Additionally, serine 48 is followed by glutamine and arginine in bovine, human and

porcine PTH (Brewer and Ronan, 1970, Keutmann *et al*, 1978, Sauer *et al*, 1974). Together these residues make a PKC substrate consensus sequence of the form S-x-R (Kemp and Pearson, 1990). The finding that PKC purified from bovine parathyroid was capable of phosphorylating bovine PTH suggests the possibility that this phosphorylation may take place *in vivo*.

PTH is phosphorylated *in vivo* under normocalcemic conditions at a ratio of about 1P : 6PTH, but the kinase responsible for this phosphorylation remains unknown (Rabbani *et al*, 1984). PTH phosphorylation occurs in an early stage in the processing of PTH, as the PTH precursor, proPTH is also phosphorylated (Rabbani *et al*, 1984). Other peptide hormones are also phosphorylated during or shortly after translation (Eiper and Mains, 1982). It has been suggested that phosphorylation may alter the glycosylation, or the cleavage pattern of the precursor peptide of secreted peptide hormones (Bennet *et al*, 1981, Browne *et al*, 1981, Noel and Mains, 1991).

The present finding that both bovine and human PTH are phosphorylated by PKC at the same site, and the finding that this site is highly conserved suggests a possible physiological role of PKC-mediated PTH phosphorylation. Because PKC appears to be involved in both secretion and degradation of PTH (Tanguay *et al*, 1991, Watson *et al*, 1992), it is possible that direct phosphorylation by PKC alters the storage and/or degradation of PTH.

Phosphorylation is known to affect the degradation of several proteins by calpain (Litersky and Johnson, 1992, Salamino *et al*, 1992, Melloni and Pontremoli, 1989, Johnson and Foley, 1993, Elvira *et al*, 1993, Elvira *et al*, 1994). The calpains are a family of calcium-activated neutral proteases (reviewed by Suzuki *et al*, 1987, Meloni and Pontremoli, 1989, and Murachi,

1989). At least six members of this family have been identified, and calpain involvement has been demonstrated in a large variety of physiologically important functions (reviewed by Suzuki *et al*, 1992, Sorimachi *et al*, 1994, and Saido *et al*, 1994). Calpain is capable of catalyzing limited proteolysis of a number of proteins *in vitro*, but does not appear to be a generalized degraditive enzyme within the cell (Wang *et al*, 1989). Endogenous calpain substrates typically contain one or more regions which are rich in proline (P), glutamic acid (E), aspartic acid (D), serine (S) and threonine (T), also termed PEST sequences (Rechsteiner, 1991). It has been suggested that threonine and serine residues in these PEST sequences may be phosphorylated, producing a highly negative region which may bind calcium (Rogers *et al*, 1987). This localization of calcium ions could then serve to activate calpain resulting in the cleavage of proteins in the vicinity of the PEST sequence (Rogers *et al*, 1987, Wang *et al*, 1989). Phosphorylation is associated with increased susceptibility to calpain cleavage of red cell membrane proteins (Salamino *et al*, 1992), as well as cytoskeletal proteins (reviewed by Melloni and Pontremoli, 1989). However, several studies indicate that phosphorylation of other proteins results in increased resistance to calpain cleavage (Litersky and Johnson, 1992, Johnson and Foley, 1993, Elvira *et al*, 1993, Elvira *et al*, 1994). Since PKC-mediated phosphorylation has been implicated in two of these studies (Elvira *et al*, 1993, Elvira *et al*, 1994), it is possible that the phosphorylation of PTH by PKC observed in the current study serves to alter its cleavage in the parathyroid cell. However this possibility remains to be examined.

Two forms of calpain have recently been identified in bovine parathyroid (Watson *et al*, 1995). Due to similarities in the HPLC elution of

PTH fragments produced by *in vitro* calpain cleavage and those secreted by parathyroid cells, it has been suggested that calpain is responsible for the degradation of PTH within the parathyroid (Watson *et al*, 1995). Analysis of the primary sequence of PTH reveals a PEST sequence within this hormone, in proximity to the observed calpain cleavage sites, which were found between residues 46 and 47 and residues 66 and 67 (Watson *et al*, 1995). The present identification of a PKC phosphorylation site one residue from an identified calpain cleavage site suggests the possibility that PKC phosphorylation may alter the cleavage position and/or the rate of cleavage of PTH.

In this study, both human and bovine PTH were phosphorylated by purified PKC at serine 48. The phosphorylation of this residue, which is highly conserved in the primary structure of PTH from several species, suggests a physiological significance for PKC-mediated PTH phosphorylation. In addition to the possibility that phosphorylation alters proteolytic degradation of PTH, other potential roles of phosphorylation can be suggested. These include potential alterations in the processing and/or storage of PTH and the biological activity of secreted PTH.

4.3.2 Further studies on the phosphorylation of PTH

In order for PKC-mediated PTH phosphorylation to be considered physiologically important, phosphorylation at the identified site must be demonstrated *in vivo*. Labeling studies in which parathyroid tissue slices or cells are incubated in media containing radioactive orthophosphate should be performed. After sufficient time for incorporation of radioactivity, PTH can be purified and the site of phosphate incorporation can be determined. If phosphorylation of serine 48 occurs *in vivo*, studies on the physiological

significance of this phosphorylation should be performed. These include analysis of the effects of phosphorylation on calpain cleavage, and examination of the extent of PTH phosphorylation in response to various treatments.

Summary

1) The PKC isoenzymes cPKC α, cPKC β, nPKC ε, aPKC ζ were identified in the parathyroid by Western blot analysis. Another aPKC polypeptide, which may be either the translation product of an mRNA splice variant of aPKC ζ, aPKC ζ', or aPKC λ,was also identified in the parathyroid. Western blotting using isoenzyme-specific antibodies failed to identify nPKC δ, nPKC η and nPKC θ in parathyroid cells harvested from normocalcemic conditions.

2) Hydroxylapatite chromatography of partially purified bovine parathyroid PKC also identified cPKC α, and cPKC β, in the parathyroid. PKC γ was not found using this method, suggesting that this isoenzyme is not present in the parathyroid.

3) Association of cPKC α with the particulate fraction of treated parathyroid tissues was found to occur only under conditions of low calcium-stimulated secretion. The particulate fractions of all treated parathyroid tissue were found to contain PKC β. The relative amount of particulate PKC β was positively correlated with the relative PTH secretion for each treatment.

4) PKC purified from both bovine parathyroid and bovine brain phosphorylated bPTH *in vitro*. Serine 48 was phosphorylated by purified bovine brain PKC in both bPTH and hPTH.

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