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THE ROLE OF CYCLIC AMP AND ITS RELATIONSHIP TO PARATHYROID
HORMONE RESPONSE IN AN *IN VITRO* MODEL OF CHONDROGENESIS

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RELATIONSHIP TO PARATHYROID HORMONE RESPONSE
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A
THESIS

Presented to the Faculty of
The University of Texas Graduate School of Biomedical Sciences
at San Antonio

in Partial Fulfillment
of the Requirements
for the Degree of
MASTER OF SCIENCE

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DEDICATION

This thesis is dedicated to my wife, Wendy, whose unending patience, sacrifice and understanding, underlie any success that I may achieve. I also dedicate this to my sons, Eric and Mark, who have continually reminded me of those things truly important.

THE ROLE OF CYCLIC AMP AND ITS
RELATIONSHIP TO PARATHYROID HORMONE RESPONSE
IN AN *IN VITRO* MODEL OF CHONDROGENESIS

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Although previous studies have established cartilage as a target tissue for parathyroid hormone (PTH), it is not clear how the state of chondrocyte maturation influences the expression of hormonal effects. In addition, PTH has been shown to both

inhibit and accelerate chondrocyte differentiation. Although cyclic AMP (cAMP) is considered the primary mediator of PTH-induced effects on various target cells, the precise role of cAMP-mediated stimulation in chondrocytes is not clear. Numerous studies demonstrating transient increases in cellular cAMP levels shortly after exposing chondrocytes to PTH suggest a mediatory role for this secondary messenger. Additional evidence is provided by the ability of cAMP analogs such as N_ε-monobutyl cAMP and N_ε,O_{2'}-dibutyl-adenosine 3',5'-cyclic-monophosphate to mimic certain effects of PTH when incubated with chondrocytes *in vitro*. Nevertheless, recent evidence suggests cAMP is not the only mediator coupling PTH to cellular responses in chondrocytes.

This study examined the effect of bovine parathyroid hormone 1-34 (bPTH) on an important marker enzyme of chondrocyte maturation, alkaline phosphatase (ALPase), in an *in vitro* model of chondrogenesis. In addition, chondrocytes were incubated with 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, and forskolin, an adenylate cyclase activator, to examine the hypothesis that regulation of ALPase activity by bPTH in cartilage cells is mediated by cyclic AMP.

Confluent, fourth passage cultures of growth zone and resting zone chondrocytes from the costochondral cartilage of 125 gram rats were incubated in DMEM, 10% fetal bovine serum and ascorbic acid containing: (1) vehicle; (2) 10⁻⁷ to 10⁻¹²M bPTH; (3) forskolin and IBMX; or (4) 10⁻⁷ to 10⁻¹²M bPTH with forskolin and IBMX, at 37°C for periods of 5 minutes, 10 minutes, and 1, 3, 12, 24, 36 and 48 hours. ALPase specific activities were then

determined in the cell layer and isolated matrix vesicle and plasma membrane fractions.

Incubation of growth zone chondrocytes with bPTH resulted in biphasic, dose-dependent stimulation of ALPase specific activity in the cell layer from 5 to 180 minutes and 36 to 48 hours. Incubation of resting zone chondrocytes with bPTH resulted in dose-dependent stimulation of ALPase activity in the cell layer from 5 to 60 minutes. Incubation of growth zone cell cultures with IBMX and forskolin resulted in biphasic stimulation of ALPase activity in the cell layer from 5 to 10 minutes and 36 to 48 hours. Incubation of resting zone chondrocytes with IBMX and forskolin did not significantly affect ALPase activity of the cell layer at any time period. Incubation of growth zone chondrocytes in bPTH with IBMX and forskolin stimulated ALPase activity at 5 and 10 minutes for all concentrations of bPTH. Incubation of resting zone cell cultures in bPTH with IBMX and forskolin did not significantly affect ALPase activity at any time period. ALPase activity in matrix vesicle and plasma membrane fractions from growth and resting zone chondrocytes incubated in bPTH with or without IBMX and forskolin generally reflected observations of the respective cell layer lysates at 10 minutes and 48 hours.

Results of this study suggest bPTH stimulates ALPase activity in growth and resting zone chondrocytes and that this stimulation is influenced by the state of cell maturation. While cAMP does not appear to mediate this effect, previous studies suggest signal transduction may involve effects on

calcium channels in a cAMP-independent manner. This possibility warrants further study and is presently being examined by this laboratory.

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I. INTRODUCTION AND LITERATURE REVIEW

Bone is a complex tissue composed of a calcified matrix and a number of cell types in various stages of differentiation. These cell stages are in delicate equilibrium with each other and influenced by a number of factors. Large numbers of hormones [e.g. parathyroid hormone (PTH), calcitonin, 1,25-dihydroxyvitamin D₃, growth hormone, corticosteroids and insulin], humoral factors (epidermal growth factor, tumor-derived growth factor, platelet-derived growth factor), and locally produced and locally active factors, have been observed to profoundly effect bone metabolism (Nijweide et al. 1986). Understanding the influence of these factors on bone and cartilage cell equilibria and differentiation will enhance our understanding of bone metabolism in health and disease.

A. Parathyroid Hormone

Native parathyroid hormone (PTH) is a linear polypeptide composed of 84 amino acids with a molecular weight of approximately 9300 daltons. Production of PTH is stimulated by any decrease in blood calcium, and conversely inhibited by increases in serum calcium. Although precise mechanisms operating in the calcium regulation of PTH remain elusive, MacGregor et al. (1988) concluded that secretory adaptation to chronic hypocalcemia involves alterations in both the synthesis and degradation of the hormone.

PTH is the principal minute-to-minute regulator of extracellular fluid calcium, acting to raise extracellular fluid calcium through actions on several organs. Coordinated action of

PTH on bone, kidney and intestine increase calcium flow to the extracellular fluid and ultimately increase blood calcium levels. Primary actions of the hormone are to: (1) increase the dissolution of bone mineral; (2) decrease renal clearance of calcium; and (3) indirectly increase calcium absorbance efficiency in the small intestine. Rapid-action regulation of extracellular fluid calcium is primarily exerted through bone and renal effects. The relative importance of these two pathways has not been resolved.

1. Mode of action. Although certain hormones exert their effects by entering the cell, similar mechanisms have not been demonstrated for PTH. Accordingly, current observations regarding the mechanism(s) by which PTH exerts cellular effects are based upon interactions with a specific cell-surface receptor. The original report of specific PTH binding was by Sutcliffe et al. (1973). Expression of critical PTH effects on bone and kidney through hormone-specific receptors on the plasma membrane of target tissue cells has since been demonstrated by a number of investigators (Karpf et al. 1987, 1988; Shigeno et al. 1988; Yamamoto et al. 1988).

It has been proposed that the interaction of hormone and receptor initiates a cascade of intracellular events, including generation of cyclic AMP, phosphorylation of proteins, intracellular entry of calcium, stimulation of the polyphosphoinositol pathway, and activation of intracellular enzymes and transport systems, leading to a number of metabolic effects. It is not clear if receptors on different target

tissues are identical in structure and binding requirements. Observations by Takano et al. (1985) suggest there is remarkable similarity among kidney, bone, and cartilage with respect to the structural requirements necessary for agonist and antagonist activities of fragments and analogs of PTH. Nevertheless, recent evidence suggests that regardless of surface receptor properties, the cellular response to PTH binding may vary from tissue to tissue.

Because most *in vitro* and *in vivo* assays indicate full biologic activity of the native PTH (1-84) molecule resides in the (1-34) fragment (Potts et al. 1982), (1-34) PTH is assumed to possess much of the structure required for activation of PTH-responsive systems. Although previous studies suggest much of this (1-34) PTH fragment exists as a random coil, Smith et al. (1987) have demonstrated it has considerable secondary and tertiary structure in solution. Frelinger and Zull (1986) determined methionine residues at positions 8 and 18 are especially important in the biologically active conformation of PTH, even though there is no evidence these residues are directly involved in receptor binding. Because the synthetic fragment bPTH (3-34) is biologically inactive but competes with bPTH (1-34) for binding to bone, the receptor binding site appears to reside within the bPTH (3-34) fragment (Wong 1986). Rosenblatt et al. (1980) and Kremer et al. (1982) found the (26-34) sequence to be of particular importance in receptor binding, while Nussbaum et al. (1980) have shown an additional but less significant binding domain between residues 10 and 15.

Despite the importance of the (1-34) sequence, evidence exists that some actions of native PTH are not shared by the N-terminal fragment alone. Included among these effects are certain stages of bone demineralization (Herrmann-Erlee et al. 1976), promotion of distal-tubular calcium transport (Puschett et al. 1984), inhibition of hematopoiesis, and stimulation of hepatic glucose release (Meytes et al. 1981). Somjen et al. (1990) found a functional domain in the central part (hPTH 24-48) of the molecule exerting mitogenic-related effects on osteoblast- and chondroblast-like cells in a cyclic AMP-independent manner. Since cyclic AMP activation by PTH has been shown to produce bone resorption, the authors speculated mid-region fragments may prove suitable for *in vivo* use in inducing bone formation without concomitant resorption. Other studies have indicated the (1-34) peptide is less effective than native hormone in competing for binding sites (Zull et al. 1977, 1980), suggesting additional binding sites may exist beyond residue 34. Such findings were confirmed by Rizolli et al. (1983) who demonstrated the presence of two binding sites for native PTH in osteosarcoma cells; one of high affinity associated with activation of adenyl cyclase, and a second of lower affinity. Rao and Murray (1985) showed this second site to be specific for the C-terminal (53-84) fragment of the peptide. Thus, while our understanding of the biologic function of C-terminal segments remains incomplete, specific binding proteins for this region do exist. It is not known if the two receptors represent separate proteins, or sub-domains of the same protein, nor the relative significance of each in

different target tissues. Nevertheless, the concept of distinct receptors responsible for separate biological actions fits well with recent evidence indicating PTH may promote similar or opposing cellular effects mediated by different secondary messengers.

2. Skeletal effects. Because the principle target cell of PTH is located in bone, the skeletal effects of PTH have been extensively investigated. However, due to the dynamic nature of bone, and the difficulty in isolating its many cell populations, the exact nature of PTH-induced effects on this tissue remain incomplete. Nevertheless, biochemical effects can be divided into two major categories: (1) rapid catabolic effects leading to bone destruction, and (2) slower anabolic effects resulting in bone formation. The stimulation of bone resorption is by far the best documented action of PTH on skeletal tissue as it contributes to the maintenance of serum calcium levels, and bone loss associated with many pathological states. Conversely, anabolic effects have been quantitated relatively recently (Tam et al. 1982; Turnbull et al. 1983), although they were described as early as 1932 (Selye 1932).

PTH is a potent stimulator of osteoclast-mediated bone resorption both *in vitro* and *in vivo*. This appears to occur via activation of existing osteoclasts and the development of new ones. Miller et al. (1984) demonstrated PTH increases the proportion of osteoclasts with ruffled borders and expands the ruffled border in individual osteoclasts *in vivo*. Anderson et al. (1985) and Silverton et al. (1987) showed PTH also activates

and increases osteoclast carbonic anhydrase II activity, resulting in increased acidity of the microenvironment between osteoclast and bone surface, and increased bone resorption. General increases in osteoclast number and the mean number of nuclei per osteoclast have also been observed to follow PTH activation (Addison 1979; Raisz and Lorenzo 1980). Lorenzo et al. (1983) and Krieger et al. (1982) attributed these rapid increases in the number and size of osteoclasts to the fusion of local, postmitotic, mononuclear precursors. Bingham et al. (1969) correlated PTH-induced changes in osteoclast activity and number with changes in serum calcium levels *in vivo*. Feldman et al. (1980) and Raisz and Lorenzo (1980) verified this calcium was derived from bone through enhanced release of $^{45}\text{Ca}^{2+}$ into the culture medium from prelabeled fetal bones exposed to PTH.

The effect of PTH on osteoclasts appears to be indirect, as several studies have failed to demonstrate the presence of PTH receptors on these cells (Silve et al. 1982; Rouleau et al. 1986). Chambers et al. (1983, 1985) confirmed this concept in observing that introduction of PTH to isolated osteoclast cultures did not effect cell motility or bone-resorbing capacity. Such findings imply the responsiveness of osteoclasts to PTH in intact bone may be dependent on another cell. Osteoblasts have been proposed as likely candidates for this mediation as they possess PTH receptors on their cell surface (Rouleau et al. 1986), and respond morphologically (Gaillard et al. 1979) and functionally (Kream et al. 1980) to PTH *in vitro*. Further evidence arises from observations demonstrating the activation of

osteoclasts by PTH is dependent on the presence of osteoblasts (McSheehy and Chambers 1986a,b). Commonly proposed mechanisms of interaction between the osteoblast and osteoclast include (1) the production of a soluble factor derived from osteoblasts and regulating osteoclastic activity (McSheehy and Chambers 1986a,b) and (2) interaction via physical contact between the two cell types (Chambers 1982).

Another potential role of the osteoblast in PTH-mediated bone resorption may involve the exposure of bone mineral. Direct contact between the osteoclast and bone mineral appears necessary for bone resorption to occur (Chambers 1985). Chambers et al. (1984b) have shown osteoclasts do not resorb bone in the presence of an intact osteoid layer, but may do so when this layer is removed. Heath (1984) and Hamilton et al. (1984, 1985) have provided evidence supporting a role for osteoblasts in osteoid degradation through an enzymatic cascade, thereby facilitating osteoclastic contact with underlying bone mineral and osteoclast-mediated bone resorption. However, there is no direct proof that osteoclasts themselves cannot degrade osteoid or that they require an exposed mineral surface to initiate attachment and resorption *in vivo* (Marks and Popoff 1988).

The effect of PTH on proliferation, differentiation and fusion of osteoclast precursors has also received considerable attention. Nijweide et al. (1978) and Lorenzo et al. (1983) demonstrated DNA synthesis is not required for up to 4 days after PTH-induced bone resorption *in vitro*, suggesting PTH stimulates the differentiation and fusion of postmitotic osteoclast

precursors. Although these results account for short term increases in the osteoclast population, they do not explain how such increases are maintained over long periods of time. Feldman et al. (1980) using ^3H -thymidine-labeled neonatal mouse calvaria showed PTH enhanced the number of labeled osteoclast nuclei in the last 24 hours of a 48-hour culture, suggesting proliferation of osteoclast progenitors. Further studies by Lorenzo et al. (1983) demonstrated continuous labeling of organ cultures exposed to PTH resulted in increased numbers of labeled osteoclast nuclei. Thus, PTH-induced proliferation of osteoclast progenitor populations may play a vital role in maintaining the osteoclastic response once the population of local, postmitotic precursors has been depleted. Because osteoclast precursors are derived from hematopoietic mononuclear cells (Marks 1983; Chambers 1985), such effects may be the result of PTH-mediated effects on bone marrow cells (Marks & Popoff 1988).

The development of bone cell models within the last decade has greatly contributed to our understanding of osteoblastic responses to PTH; however, because many studies have been conducted using isolated osteoblast cultures or tumor cell lines possessing features of osteoblasts, results have often been inconsistent and conflicting. PTH has resulted in both inhibition (Bingham et al. 1969) and stimulation (Tam et al. 1982) of osteoblast activity. While Dietrich et al. (1976) and Kream et al. (1980) demonstrated *in vitro* that PTH inhibits osteoblastic synthesis of certain matrix proteins, other studies have indicated protein synthesis is enhanced (Yee and Sutton

1989). In addition, effects on a commonly accepted marker for the osteoblast phenotype, alkaline phosphatase, have been reported to be both inhibited (Majeska and Rodan 1982a) and stimulated (Puzas and Brand 1985) by PTH. Such conflicting results are traditionally explained in terms of differing dosages and the short-term catabolic and long-term anabolic effects of PTH on the osteoblast cell line. Short-term catabolic effects appear to involve a direct, dose-dependent inhibition of mature osteoblast activity (McGuire and Marks 1974), while PTH-induced stimulation of osteoblasts occurs only at low doses and may be the result of long-term effects on osteoblast progenitors.

Although recent studies indicate the catabolic effects of PTH involve direct interference with the osteogenic activity of osteoblasts, there is no consensus in the literature regarding the role of PTH in regulating this activity. PTH treatment of bone organ cultures (Luben and Cohn 1976) or primary cultures of osteoblasts (Luben et al. 1976) have resulted in decreased levels of citrate decarboxylation, suggesting inhibition of metabolic activity. Gaillard et al. (1979) found morphologic changes in osteoblasts exposed to PTH *in situ*. Evidence for the anabolic effects of PTH has been provided by Tam et al. (1982) and Gunness-Hey and Hock (1984) who observed increased bone apposition rates in animals treated with low doses of PTH for long periods of time. Similarly, Van der Plas et al. (1985) demonstrated PTH-induced proliferation of osteoblast-like cells *in vitro*.

The precise mechanisms mediating PTH effects on skeletal

tissue at the cellular level have not been elucidated. The concept of cyclic AMP (cAMP) as the intracellular mediator responsible for hormone action was introduced by Rall and Sutherland over 30 years ago (Rall and Sutherland 1958). The cAMP analogue, dibutyryl cAMP, causes PTH-like effects with significant cAMP stimulation. In addition, several factors (e.g. osteogenin) have been shown to modify PTH-induced cAMP responses by osteogenic cells (Vukicevic et al. 1989). However, Herrmann-Erlee et al. (1983) demonstrated significant differences between adenylate cyclase-stimulating activity and the ability to resorb bone of several PTH analogs. For example, bPTH (3-34) was shown to exert bone resorption in fetal rat calvaria without significant increases in cAMP activity. Based upon these and similar observations, a two-receptor model for the action of PTH on osteoblasts was proposed. According to this model, each receptor (site) is linked with its own second messenger; one receptor controls the activity of the adenylate cyclase system and the other the influx of Ca^{2+} in the osteoblast. A similar mechanism for PTH signal transduction in kidney cells has also been proposed (Hruska et al. 1987). Later work supported the concept that cAMP alone is not the exclusive second messenger of bone resorption, as forskolin (an adenylate cyclase activator), unlike PTH, was unable to induce sustained bone resorption, while such effects were observed when the calcium ionophore A23187 and forskolin were combined. In addition, introduction of the Ca^{2+} channel blockers verapamil and nifedipine significantly inhibited PTH-stimulated bone resorption (Herrmann-Erlee et al. 1988).

Similar observations by Reid et al. (1987) have led to the conclusion that Ca^{2+} and cAMP have specific roles as second messengers in PTH-stimulated bone resorption, and may explain the diverse anabolic and catabolic effects observed by PTH.

Increases in intracellular calcium in other systems has been shown to arise from either increased transmembrane calcium flux or increased release of intracellular calcium stores or both. Such increases activate cellular responses by several mechanisms (Rasmussen 1986). It has become apparent for a number of hormones that increases in intracellular calcium result from hormonal interaction with a membrane receptor that stimulates turnover of the polyphosphoinositol pathway. The inositol 1, 4, 5-triphosphate generated acts as an intracellular second messenger, similar to cAMP, and stimulates the release of intracellular stores of calcium, which in turn amplify a cascade of metabolic consequences (Berridge and Irvine 1984; Nishizuka 1984). Thus, PTH stimulation of polyphosphoinositol metabolism and increased intracellular flux of calcium may also represent mechanisms of PTH action (Meltzer et al. 1982); however, little is known of the details, kinetics, or physiological significance of such a non-cAMP dependent, PTH-mediated pathway. In summary, PTH-stimulated increases in intracellular calcium may act synergistically, independently, or even conversely to the effects of cAMP. A clear picture of the interplay of these potential second messengers (cAMP, polyphosphoinositols, calcium) of PTH action on bone is not yet available.

3. Cartilaginous effects. Relationships between the

osteoblast and chondrocyte are of considerable interest. Some investigators have implied the existence of a common, committed progenitor for bone- and cartilage-forming cells. Others propose the chondrocyte and osteoblast phenotype to be interconvertible under the proper circumstances. Evidence is not conclusive, and in some instances has been contradicting, as mechanisms behind the expression of bone or cartilage phenotypes remain unclear. There is insufficient evidence to assume the existence of two separate, non-interconvertible cell lines, arising from a common progenitor cell. However, current popular thought considers the osteoblastic and chondroblastic lines as distinct branches of a single osteogenic line.

Cells with a chondrogenic potential are vital to our understanding of bone growth and repair, as they can be stimulated to differentiate, calcify and support subsequent bone formation (Urist and McLean 1953). Much of what is known about the process of mineralization has been derived from studies of cartilage cells. The epiphyseal plate of long bones has provided the most extensively studied mineralization process, providing a model with discrete and easily identified cellular zones. Stages of chondrocyte differentiation and mineralization at the epiphyseal plate have been intensively investigated (Boskey 1981). Resting zone cartilage is comprised of cells in a proteoglycan rich matrix aligned into columns. Once these cells proliferate, extracellular matrix vesicles are produced. As the cells hypertrophy into growth zone cartilage, component chondrocytes possess a plasma membrane widely different from

resting cells, being particularly enriched in alkaline phosphatase (Pollesello et al. 1990). Matrix vesicles mediate the process of mineralization and were first described independently by Anderson (1967) and Bonucci (1967). These structures bud from the chondrocyte plasma membrane (Hale and Wuthier 1987) and move into the adjacent matrix. Pollesello et al. (1990) have suggested the *in vivo* formation of matrix vesicles may be mediated by Ca^{2+} -activated neutral proteases. The first signs of mineralization in cartilage occur within matrix vesicles (Anderson 1973; Vaananen et al. 1983) at some distance from the cell from which they were derived. Matrix vesicles contain phospholipids, alkaline phosphatase, alkaline ATPase, and pyrophosphatase (Wuthier 1975; Akisaka and Gay 1985). Alkaline phosphatase has been shown in previous studies to be a vital component in matrix vesicle mediated mineral formation (Register et al. 1984a, b), with the reduction of alkaline phosphatase levels in matrix vesicles expected to reduce their ability to induce mineralization. De Bernard et al. (1986) have demonstrated alkaline phosphatase to be a calcium binding glycoprotein. Phospholipids and alkaline phosphatase are believed to sequester calcium using ATPase provided energy for transport, and initiate formation of the first mineral crystals within the vesicle. Pyrophosphatases inactivate local inhibitors of mineralization, and alkaline phosphatase can further act to provide phosphate for immediate mineral formation (Ali 1983). Schwartz et al. (1988) studied isolated preparations of matrix vesicles and found that most of the increase in alkaline

phosphatase activity associated with cartilage calcification is localized to the matrix vesicles. Mineralization of adjacent collagen first occurs within and then between fibrils. Intermediate steps between vesicular and intrafibrillar mineralization are unknown. Matrix vesicles have been found in other mineralizing tissues, including bone and dentin; however, they are not nearly as numerous and do not appear near mineralizing sites in collagen.

Despite similarities between osteoblasts and chondrocytes, effects of PTH on cartilage are substantially less documented and appear to vary with the chondrocyte model used. Such differences may reflect unique characteristics of individual cartilage types *in vivo* (Takano et al. 1987). Nevertheless, studies implicating PTH in chondrocyte mitogenesis (Chin et al. 1986; Lewinson and Silbermann 1986; Pines and Hurwitz 1988; Livne 1989), cytodifferentiation (Hiraki et al. 1985; Takano et al. 1985), enhancement of activity in a rate-limiting enzyme in polyamine biosynthesis, ornithine decarboxylase (Takano et al. 1987), and enhancement of matrix synthesis (Copray et al. 1988; Levy et al. 1988) suggest cartilage is also a target organ for PTH.

Knowledge of the precise mechanisms mediating the effects of PTH on cartilage cells remain incomplete. Enomoto et al. (1989) were the first to document the physicochemical and kinetic properties of PTH receptors on chondrocytes. Using cultured rabbit costal chondrocytes, they demonstrated a single class of saturable, high affinity PTH binding sites on growth and resting zone cells. In addition, growth cells exhibited more receptors

and responded more strongly to the hormone than did resting cells; suggesting PTH-induced effects *in vivo* are regulated by the expression of specific receptors, and increases in the number of such receptors may play an important role in chondrocyte differentiation. Pines and Hurwitz (1988) confirmed the presence of specific PTH receptors on avian epiphyseal growth plate chondroprogenitor cells in demonstrating the inhibitory action of the (3-34) PTH analog. In addition, their finding that avian fibroblasts failed to react to PTH suggested cellular specificity to the peptide.

Although cAMP is considered the primary mediator of PTH-induced effects on various target cells, the precise role of cAMP-mediated stimulation in cartilage is not clear. Numerous studies demonstrating transient increases in cAMP levels shortly after exposing chondrocyte cultures to PTH suggest a mediatory role for cAMP (Takano et al. 1987; Levy et al. 1988; Pines and Hurwitz 1988; Grigoriadis et al. 1989). In addition, such responses can be potentiated by hormonal factors, such as certain vitamin D metabolites (Bradbeer 1987; Bradbeer et al. 1988), and mechanical stress (Uchida et al. 1988). Additional evidence is provided by the ability of cAMP analogs to mimic certain PTH effects when exposed to chondrocytes *in vitro* (Sullivan et al. 1984; Hiraki et al. 1985). For example, cartilage grown in medium containing N6-monobutyl cAMP (BtcAMP) demonstrates marked hypercellularity with many immature chondrocytes (Burch and Lebovitz 1981). Chin et al. (1986) exposed cultures of chicken epiphyseal growth plate hypertrophic chondrocytes to

physiological levels of PTH and found that while matrix vesicle formation per se was not significantly decreased, a decrease in the expression of cellular alkaline phosphatase accompanied by an increase in cell division and protein synthesis was observed. Because these effects were augmented by 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, and mimicked by the cAMP analogue N6,02'-dibutyryl-adenosine 3',5'-cyclic-monophosphate (DBcAMP), results indicated that effects were mediated by the classic cAMP mechanism. The authors concluded that stimulation of cell division and suppression of cellular alkaline phosphatase indicates PTH causes cells to revert to a less differentiated state. These conclusions were supported by Kato et al. (1990) finding that PTH caused 60-90% decreases in alkaline phosphatase activity and the incorporation of $^{45}\text{Ca}^{2+}$ into insoluble material, suggesting PTH-induced inhibition of the expression of mineralization-related phenotypes by growth plate chondrocytes. Thus, elevation in PTH resulting from lowered circulating levels of Ca^{2+} has been hypothesized to inhibit mineral deposition in the growth plate and provide a physiological mechanism to prevent further $\bar{\text{d}}\bar{\text{r}}\bar{\text{a}}\bar{\text{i}}\bar{\text{n}}$ on serum Ca^{2+} .

While the evidence for cAMP-mediated effects are convincing, it is increasingly apparent that other mediators are involved. Kato et al. (1988) observed that while cAMP analogs mimic some PTH-induced effects on chondrocytes, the nucleotides and PTH appear to stimulate proteoglycan synthesis through different mechanisms. Iannotti et al. (1990) found PTH increased proteoglycan synthesis of growth plate chondrocytes by 30-50%.

They postulated mediation occurred via breakdown of membrane phosphoinositides into inositol (1, 4, 5) triphosphate, resulting in increased intracellular Ca^{2+} , and activation of protein kinase C. Schluter et al. (1989) found PTH-induced mitogenic effects in primary cultures of chicken chondrocytes could not be mimicked by forskolin or dibutyryl-cAMP, both agents previously shown to induce ornithine decarboxylase in chondrocytes and enhance cAMP levels experimentally. Conversely, the authors found evidence that signal transduction of PTH in this instance may involve effects on calcium channels in a cAMP-independent manner. The authors concluded further studies were needed to determine the exact role of cAMP in the mediation of PTH effects on cartilage cells.

B. Study Objectives

The role and regulation of cartilage in endochondral ossification is paramount to our understanding of bone growth and repair. Such studies have been facilitated in recent years by the development of an *in vitro* model using chondrocytes cultured from the costochondral cartilage of rats (Boyan et al. 1988). This model establishes two populations of chondrocytes in distinct stages of differentiation, and has been successfully used to study the effects of vitamin D metabolites on plasmalemma and matrix vesicle membranes of growth zone and resting zone chondrocytes (Boyan et al. 1988). Costochondral cartilage was used in this study due to distinct cell zones and the ability to dissect more growth cartilage per unit time versus epiphyseal growth plates. Although costochondral and epiphyseal cartilages

are under different mechanical stresses *in vivo*, it is felt the benefits of the costochondral model compensate for any potential differences. Because differences in vitamin D stimulation of chondrocytes and matrix vesicles suggest an effect which is cell and metabolite specific, it is hypothesized that vitamin D, as well as other agents such as parathyroid hormone, can be used as a marker of cell differentiation *in vitro*.

The present study examined the effect of bovine parathyroid hormone 1-34 (bPTH) on alkaline phosphatase specific activity in growth and resting zone chondrocyte cultures. In addition, the hypothesis that the regulation of this activity by bPTH is mediated by cyclic AMP, was also examined. The first set of experiments studied the dose and time course response of alkaline phosphatase activity to bPTH in the cell layer of growth and resting zone chondrocytes. In addition, growth and resting zone chondrocytes were also incubated with factors known to increase cellular levels of cyclic AMP in order to examine the role of this mediator in stimulating alkaline phosphatase specific activity.

The second set of experiments examined the effect of bPTH on alkaline phosphatase specific activity in matrix vesicle and plasma membrane fractions of growth and resting zone chondrocytes, using critical times of enzyme activity determined by the first set of experiments. The role of cyclic AMP as a mediator of these effects was again studied by examining chondrocyte cultures incubated with factors known to increase cellular levels of cyclic AMP.

II. MATERIALS AND METHODS

A. Chondrocyte Cultures

Details of the chondrocyte culture system used in this study have been previously described by Boyan et al. (1988). Rib cages from 125 gram Sprague-Dawley rats were removed by sharp dissection and placed in serum-free Dulbecco's modified Eagle's medium (DMEM). Ribs were then separated, defleshed, and the resting zone and adjacent growth zone cartilages removed. After completing the dissections, cartilages were separated according to their zone of origin, taking care to dissect out intervening tissue to limit cross contamination of cell zones. Due to the time required to complete dissections, cartilage slices were placed in serum-free DMEM and incubated overnight in an atmosphere of 5% CO₂ at 37° C. The DMEM was then replaced by two 20-minute washes of Hank's Balanced Salt Solution (HBSS), followed by sequential incubations in 1% trypsin (Gibco, Grand Island, NY) for one hour and 0.02% collagenase (Worthington type II, Freehold, NJ) for three hours (Suzuki et al. 1981). All enzymes were prepared in HBSS. Once enzymatic digestion of the extracellular matrix was complete, cells and tissue debris were separated by filtration through sterile 40 mesh nylon and collected from the filtrate by centrifugation at 500 X g for ten minutes, resuspended in DMEM and counted. Cell viability using this protocol is greater than 90% using Trypan blue dye exclusion. Cells were plated in 25 mm culture dishes at initial densities of 10,000 cells/cm² for resting zone cells or 25,000 cells/cm² for growth zone chondrocytes (Rifas et al.

1982). Cells were incubated in DMEM containing 10% fetal bovine serum and 50 ug/ml vitamin C in an atmosphere of 5% CO₂ at 37°C and 100% humidity for 24 hours. Culture media was replaced at 24 hours initially, and then at 72 hour intervals until cells reached confluence. Upon confluence, cells were subcultured to T-75 flasks at the same plating densities and allowed to return to confluence. Subculturing was performed a maximum of three passages to ensure retention of a differential phenotype. Both cell populations took 5-8 days (7 day average) to reach confluence in primary culture and at each subculture.

Bovine parathyroid hormone 1-34 (bPTH) (Calbiochem Co., La Jolla, CA) was tested at physiological and pharmacological doses ranging from 10⁻⁷ to 10⁻¹²M with and without IBMX (Chin et al. 1986) and forskolin (Farnsdale et al. 1988). Each experiment included control cultures that contained the highest concentration used as the hormone and/or IBMX and forskolin vehicles. All experiments examining the effect of bPTH with or without IBMX and forskolin on the cell layer were conducted in 24 well plates using the following protocol: Fourth passage, confluent cultures of growth and resting zone chondrocytes were incubated in DMEM containing (1) vehicle; (2) 10⁻⁷ to 10⁻¹²M bPTH; (3) forskolin and IBMX; or (4) 10⁻⁷ to 10⁻¹²M bPTH with forskolin and IBMX, at 37°C for periods of 5 minutes, 10 minutes, and 1, 3, 12, 24, 36 and 48 hours prior to harvest.

B. Preparation of Cell Layers

Cell layers were prepared following the method of Hale et al. (1986). After incubation with the specified factor for the

predetermined time increment in 24-well culture dishes (Corning, NY), the medium was decanted and the cell layer washed two times with phosphate buffered saline (PBS). Cells were then removed using a cell scraper. After centrifugation, the cell layer pellet was washed two times with PBS and resuspended by vortexing in 500 μ l deionized water plus 25 μ l 1% Triton X-100. Enzyme assays were performed using the lysates of cell layers.

C. Preparation of Cell Fractions

Experiments examining alkaline phosphatase specific activity in chondrocyte matrix vesicles and plasma membranes included the same experimental groups used to examine the enzyme activity of cell layers. The preparation of chondrocyte matrix vesicles and plasma membranes has been previously described by Boyan et al. (1988). Chondrocyte cultures were washed twice with serum-free DMEM, trypsinized (1% in HBSS), and the reaction stopped with DMEM containing 10% FBS. Cells were then collected by centrifugation at 500 X g for 10 minutes. Cell pellets were resuspended in saline and counted using an automated cell counter. The remaining sample was washed in 0.9% saline and centrifuged at 500 X g for ten minutes, with pellets resuspended and stored in 0.25 sucrose. Supernatant from the trypsin digest was centrifuged at 11,750 X g for 20 minutes to pellet a mitochondria/membrane fraction, and for one hour at 34,000 X g to pellet matrix vesicles.

Plasma membranes were prepared from homogenates of the cell pellets using a Tenbroek homogenizer and differential centrifugation, followed by sucrose density centrifugation

(Fitzpatrick et al. 1969). Resulting membranes were resuspended in 0.9% NaCl and stored. All samples used in subsequent assays represent the combination of two cultures (i.e. two T-75 flasks). The protein content of each fraction was determined by a miniaturization of the method of Lowry et al. (1951).

D. Enzyme Assays

Enzyme specific activities were assayed in both the cell layer lysate and matrix vesicle/plasma membrane fractions. Alkaline phosphatase [orthophosphoric monoester phosphohydrolase alkaline (EC 3.1.3.1)] was measured as a function of release of para-nitrophenol from para-nitrophenylphosphate at pH 10.2 (Bretandiere and Spillman 1984). Data are expressed in terms of Pi produced to facilitate comparisons with previous studies (Schwartz et al. 1988; Boyan et al. 1988; Schwartz and Boyan 1988). There is a one to one correlation of umoles Pi produced with umoles para-nitrophenol produced.

E. Statistical Analysis

Each experiment was conducted a minimum of two times. Data presented are from typical experiments. Each data point represents the mean \pm SEM for six samples. Statistical significance was determined by comparing each data point to the control (containing the vehicle for bPTH and/or the vehicle for IBMX and forskolin) using Student's t test. Significance of differences were evaluated by analysis of variance. No differences in response were observed when the control with vehicle was compared to the control without vehicle.

III. RESULTS

A. Effect of bPTH on Alkaline Phosphatase Specific Activity

Incubation of growth zone chondrocytes with bPTH resulted in biphasic, dose-dependent stimulation of alkaline phosphatase (ALPase) specific activity (Figures 1 and 2) from 5 to 180 minutes and 36 to 48 hours. Peak stimulation occurred at 10 minutes and 48 hours, with 4 to 6-fold increases in enzyme activity observed. Examination of ALPase activity in growth zone matrix vesicle and plasma membrane fractions at peak times of enzyme activity in the cell layer revealed dose-dependent stimulation of ALPase activity for matrix vesicles isolated from chondrocytes incubated in bPTH for 10 minutes (Figure 3). This increase peaked at a bPTH concentration of $10^{-10}M$ and was approximately 2-fold in magnitude. Conversely, dose-dependent inhibition of ALPase activity was observed for plasma membranes isolated from chondrocytes incubated in bPTH for 10 minutes. Dose-dependent increases in ALPase activity were recorded for matrix vesicles and plasma membranes isolated from chondrocytes incubated in bPTH for 48 hours (Figure 4).

Incubation of resting zone chondrocytes with bPTH resulted in dose-dependent stimulation of ALPase specific activity at 5, 10 and 60 minutes, with a return to baseline values observed at 180 minutes (Figures 5 and 6). Examination of ALPase activity in matrix vesicle and plasma membrane fractions isolated from chondrocytes incubated in bPTH for 10 minutes revealed 2-fold, dose-dependent stimulation of enzyme activity in matrix vesicles, and no significant effect at any concentration of bPTH for plasma

membranes (Figure 7). ALPase activity in matrix vesicle and plasma membrane fractions isolated from chondrocytes incubated in bPTH for 48 hours was not significantly affected (Figure 8).

B. Effect of IBMX and Forskolin on Alkaline Phosphatase Specific Activity

Incubation of growth zone chondrocytes with IBMX and forskolin resulted in biphasic stimulation of ALPase activity in the cell layer at 5 and 10 minutes, and 36 and 48 hours (Figures 9 and 10). Peak stimulation occurred at 5 minutes, resulting in a 4-fold increase in enzyme activity. 6- and 2-fold increases in ALPase activity were observed for matrix vesicle and plasma membrane fractions respectively when isolated from chondrocytes incubated with IBMX and forskolin for 10 minutes (Figure 13).

Incubation of resting zone chondrocytes with IBMX and forskolin did not significantly affect ALPase specific activity in the cell layer at any time period (Figures 11 and 12). Similarly, matrix vesicle and plasma membrane fractions isolated from chondrocytes incubated with IBMX and forskolin for 10 minutes did not demonstrate significant differences in enzyme activity when compared to the control (Figure 13).

C. Effect of bPTH with IBMX and Forskolin on Alkaline Phosphatase Specific Activity

Incubation of growth zone chondrocytes in bPTH with IBMX and forskolin revealed significant stimulation of ALPase specific activity in the cell layer at 5 and 10 minutes for all concentrations of bPTH (Figures 14 and 15). Peak stimulation was observed at 10 minutes, resulting in a 4 to 5-fold increase in enzyme activity. Matrix vesicle and plasma membrane fractions

isolated from chondrocytes incubated in bPTH with IBMX and forskolin for 10 minutes demonstrated enhanced ALPase activity for both cell fractions at all concentrations of bPTH examined (Figure 16).

Incubation of resting zone chondrocytes in bPTH with IBMX and forskolin did not significantly affect ALPase specific activity in the cell layer at any time period (Figure 17). However, dose-dependent stimulation of enzyme activity was observed for matrix vesicle and plasma membrane fractions isolated from chondrocytes incubated with the three agents for 10 minutes (Figure 18).

Figure 1:

The effect of bPTH (1-34) on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

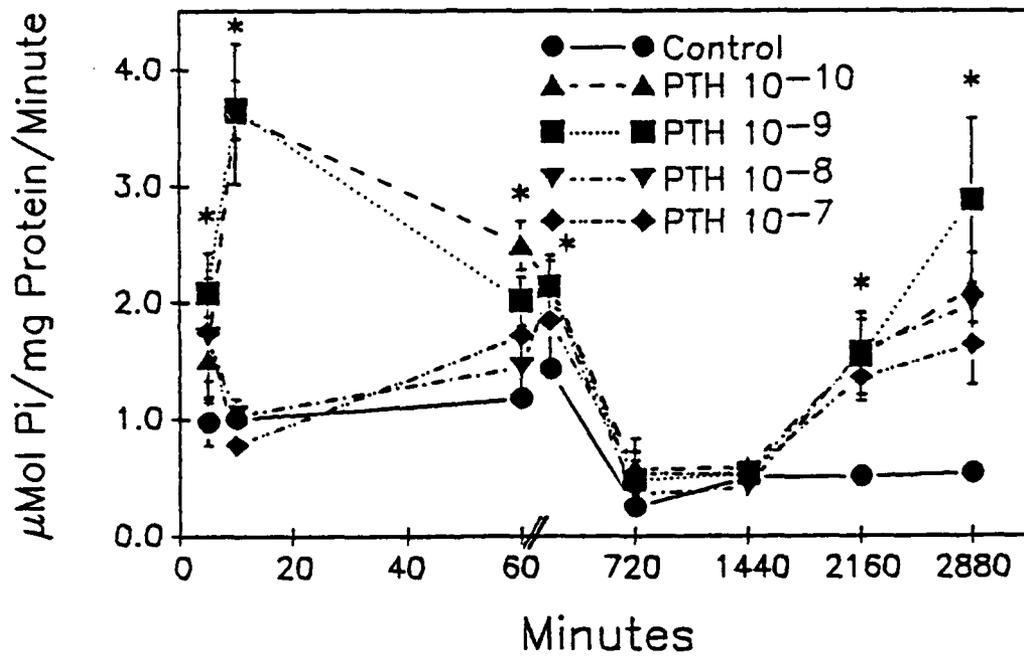


Figure 2:

The effect of bPTH (1-34) on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

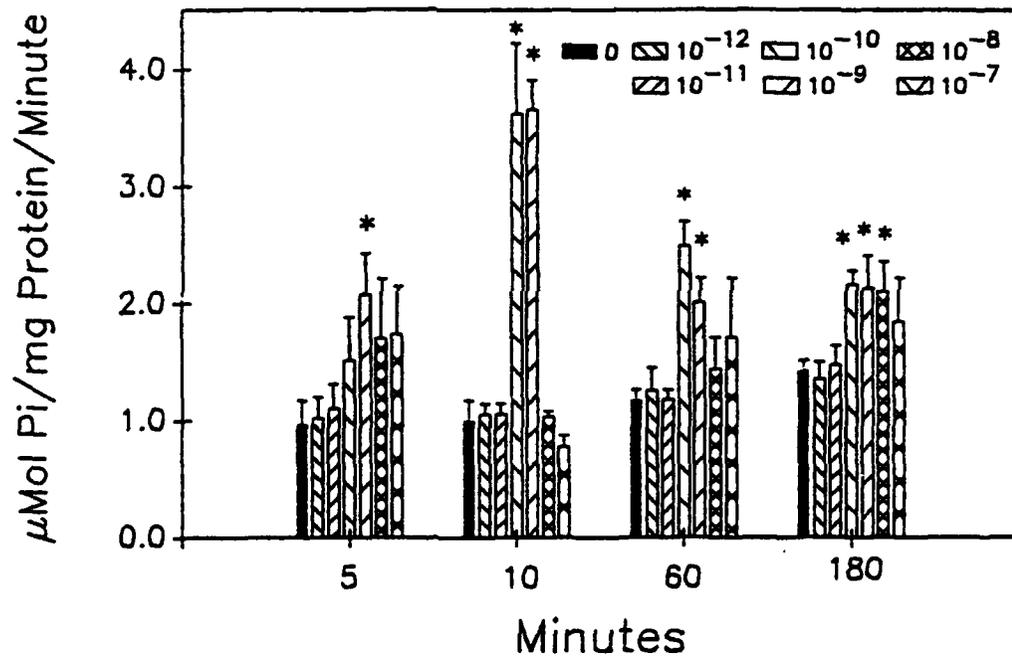


Figure 3:

The effect of bPTH (1-34) on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth zone chondrocytes at 10 minutes.

Matrix vesicles and plasma membranes isolated from two T-75 flasks were combined for each sample. Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

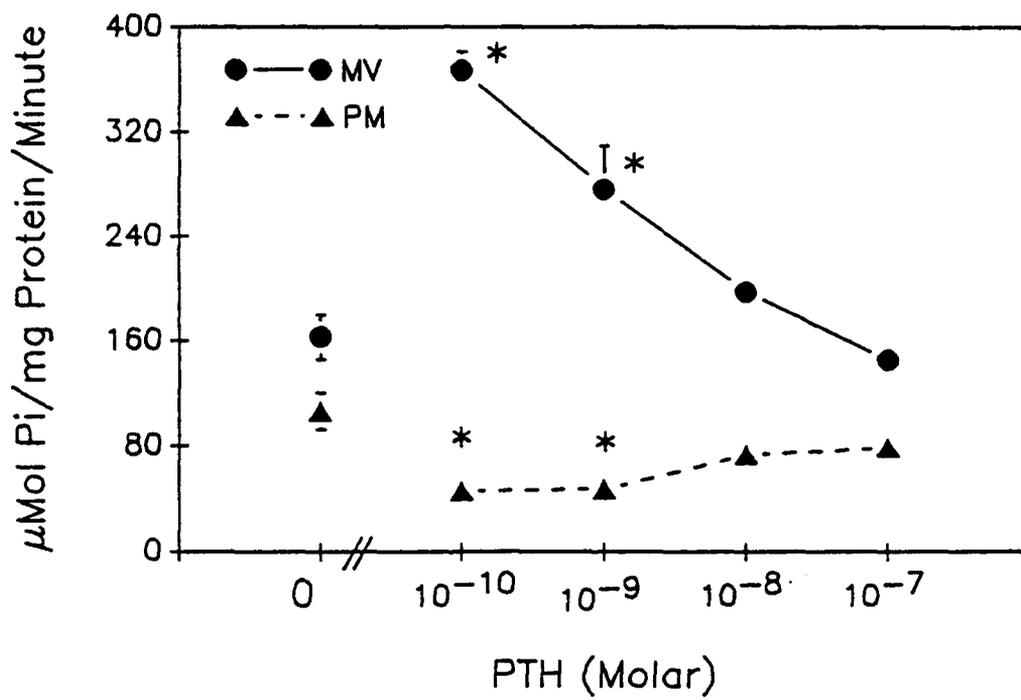


Figure 4:

The effect of bPTH (1-34) on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth zone chondrocytes at 48 hours.

Matrix vesicles and plasma membranes isolated from two T-75 flasks were combined for each sample. Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

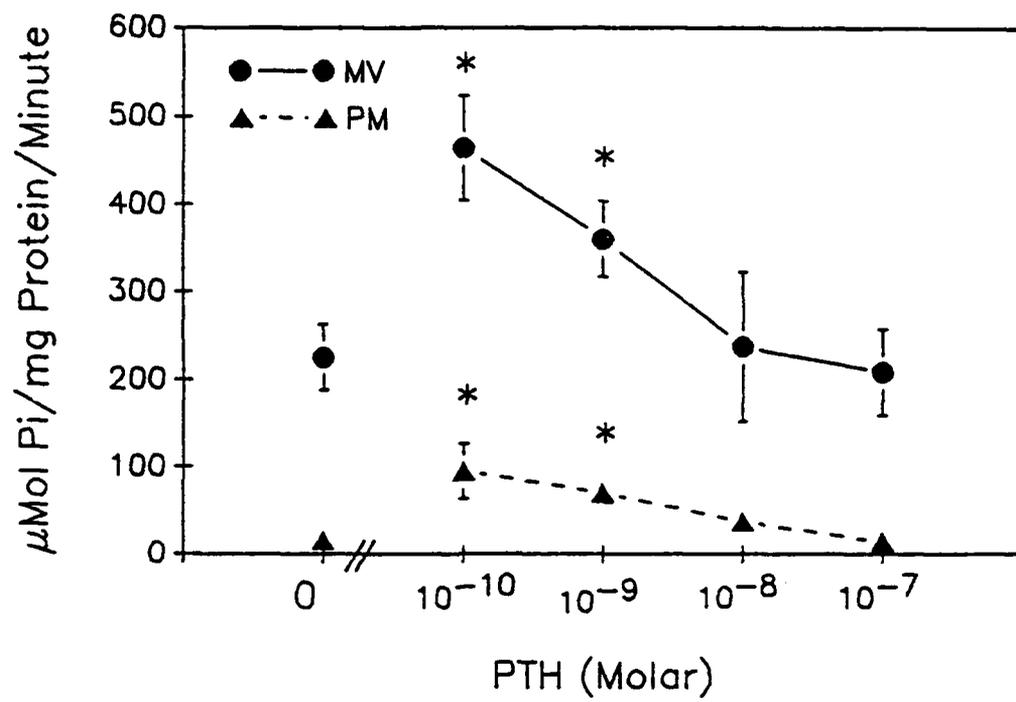


Figure 5:

The effect of bPTH (1-34) on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral resting zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

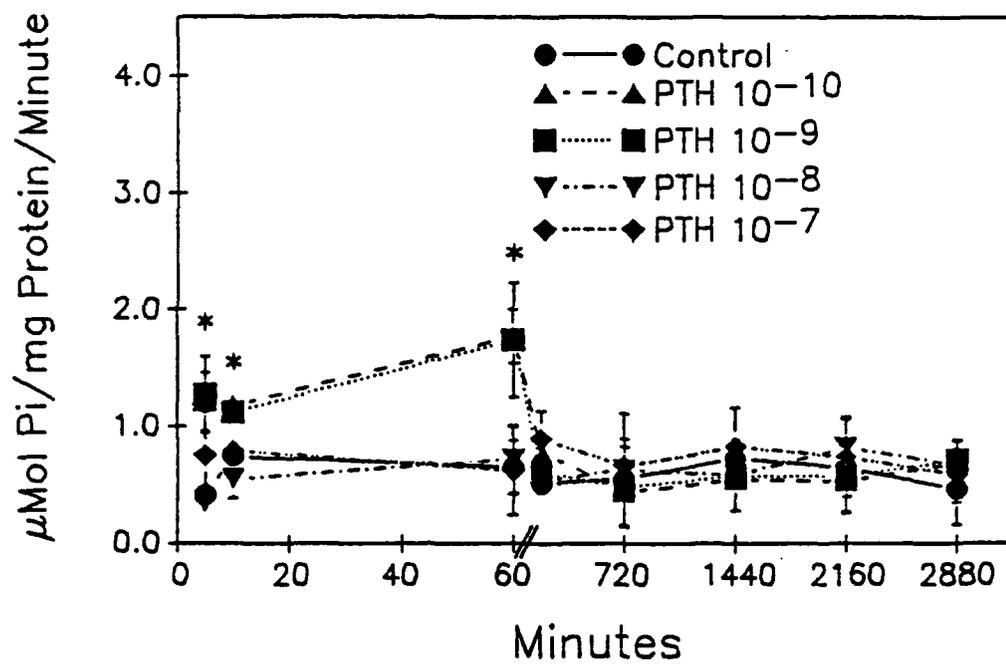


Figure 6:

The effect of bPTH (1-34) on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral resting zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

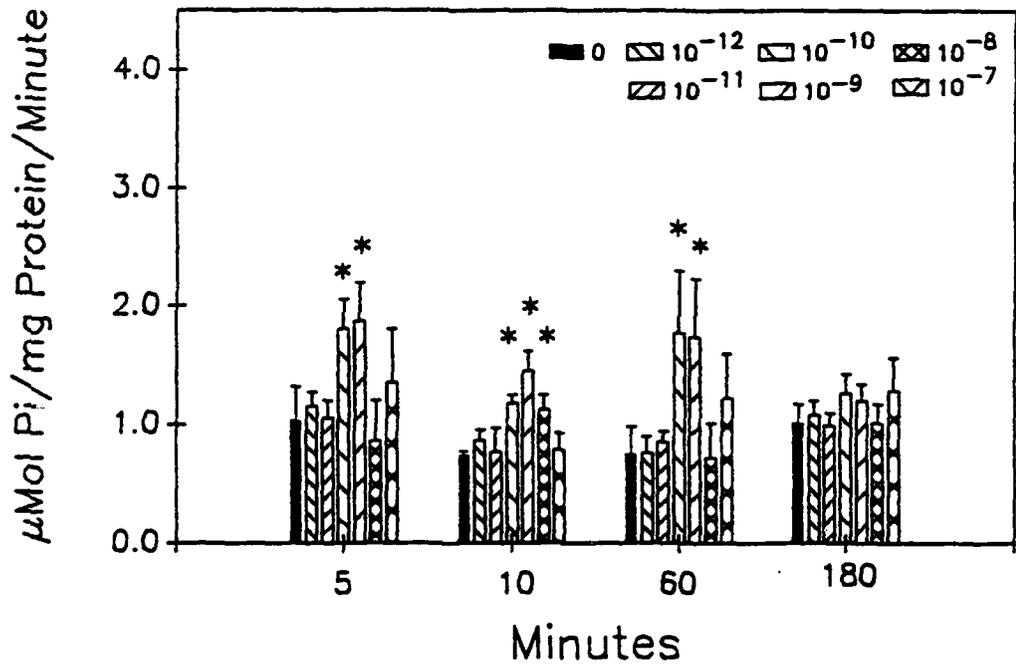


Figure 7:

The effect of bPTH (1-34) on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral resting zone chondrocytes at 10 minutes.

Matrix vesicles and plasma membranes isolated from two T-75 flasks were combined for each sample. Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

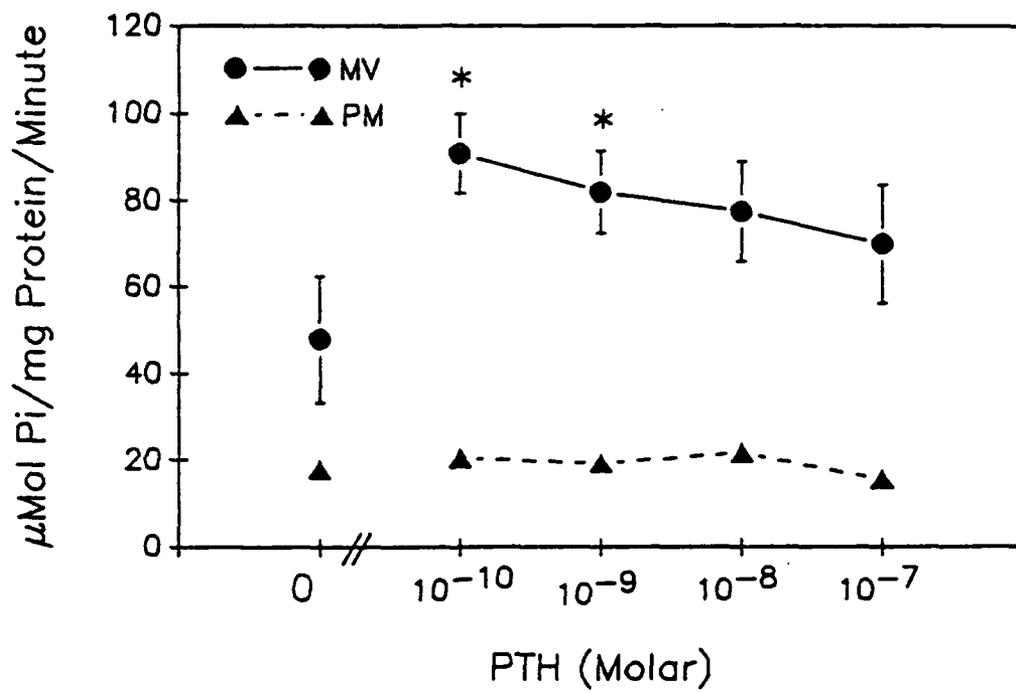


Figure 8:

The effect of bPTH (1-34) on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral resting zone chondrocytes at 48 hours.

Matrix vesicles and plasma membranes isolated from two T-75 flasks were combined for each sample. Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

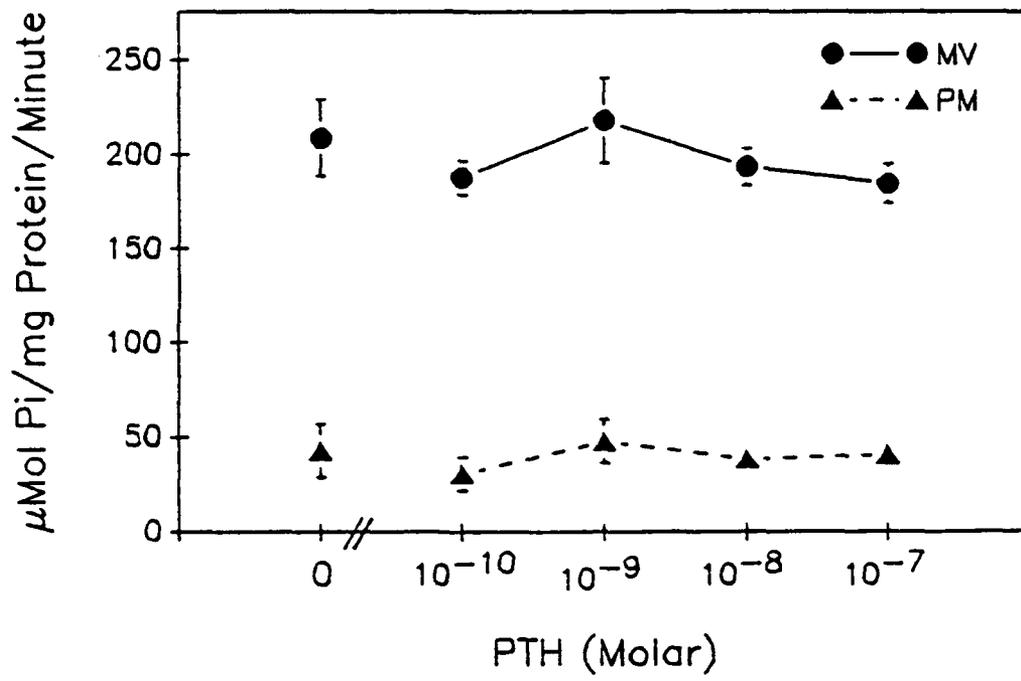


Figure 9:

The effect of IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample versus control. Data shown are from a single experiment. Each experiment was repeated two times.

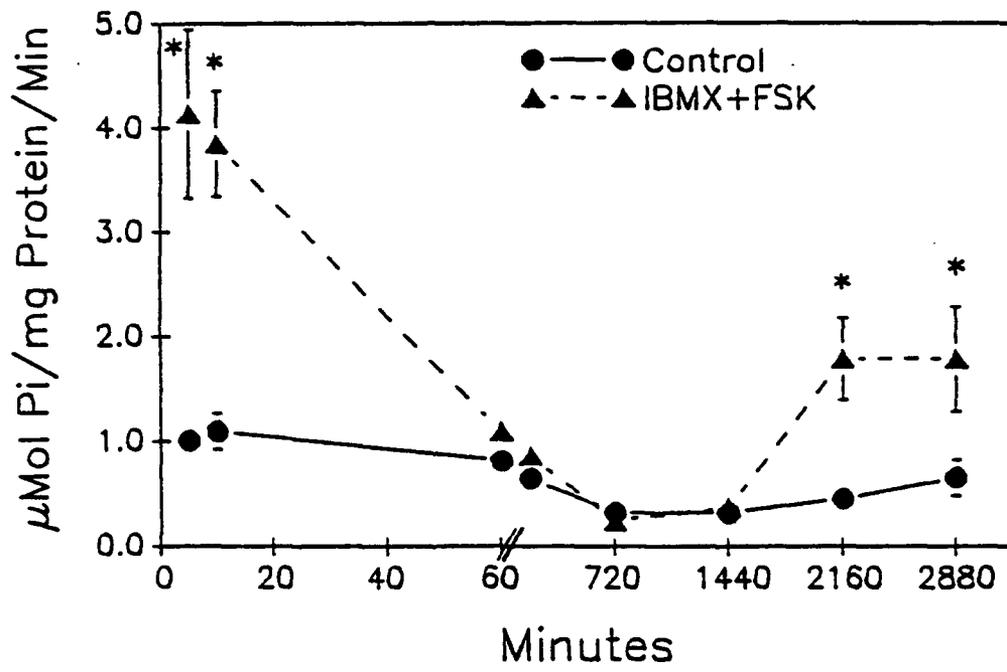


Figure 10:

The effect of IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample versus control. Data shown are from a single experiment. Each experiment was repeated two times.

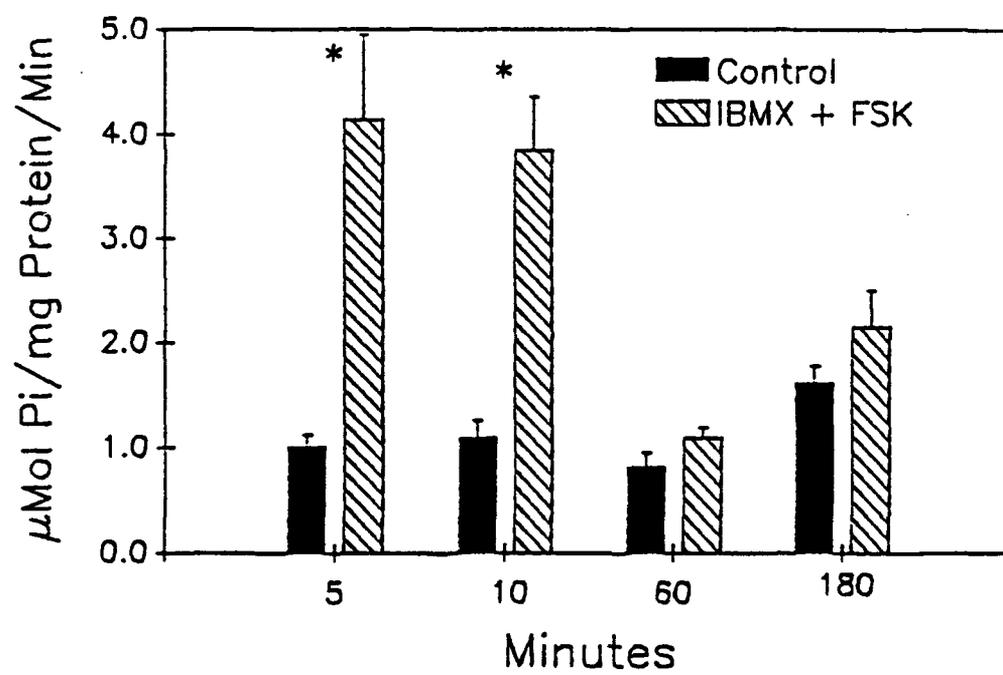


Figure 11:

The effect of IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral resting zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample versus control. Data shown are from a single experiment. Each experiment was repeated two times.

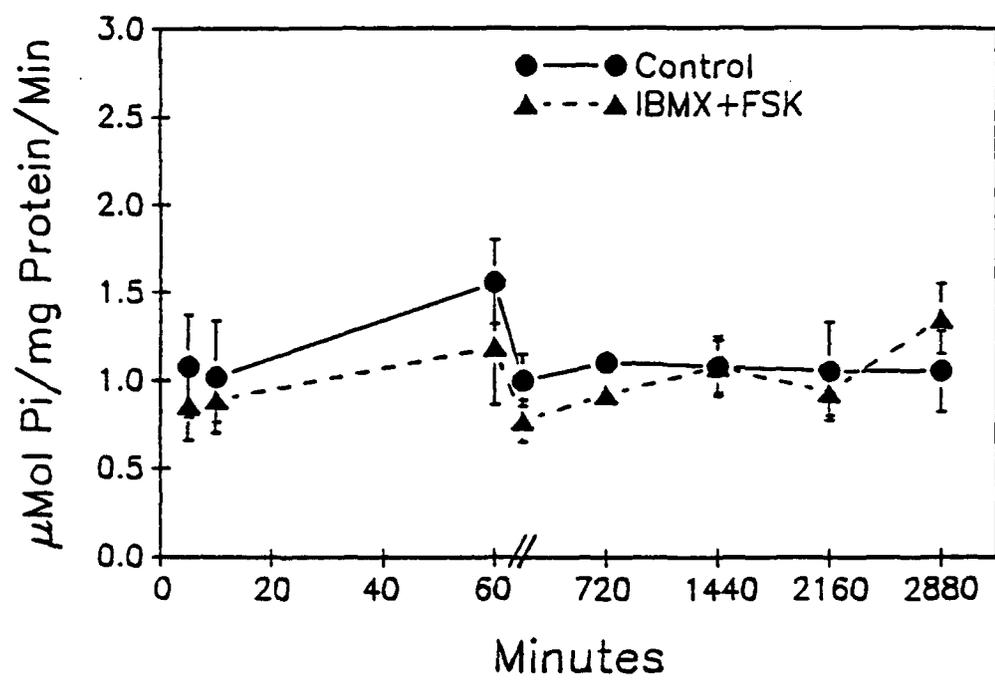


Figure 12:

The effect of IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral resting zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample versus control. Data shown are from a single experiment. Each experiment was repeated two times.

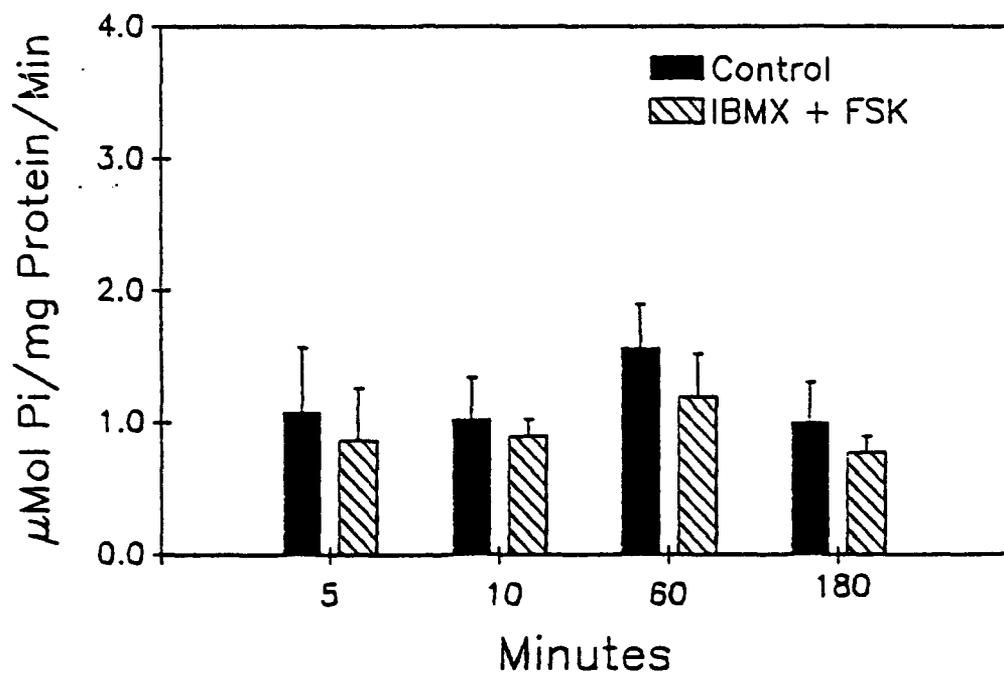


Figure 13:

The effect of IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth and resting zone chondrocytes at 10 minutes.

Matrix vesicles and plasma membranes isolated from two T-75 flasks were combined for each sample. Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

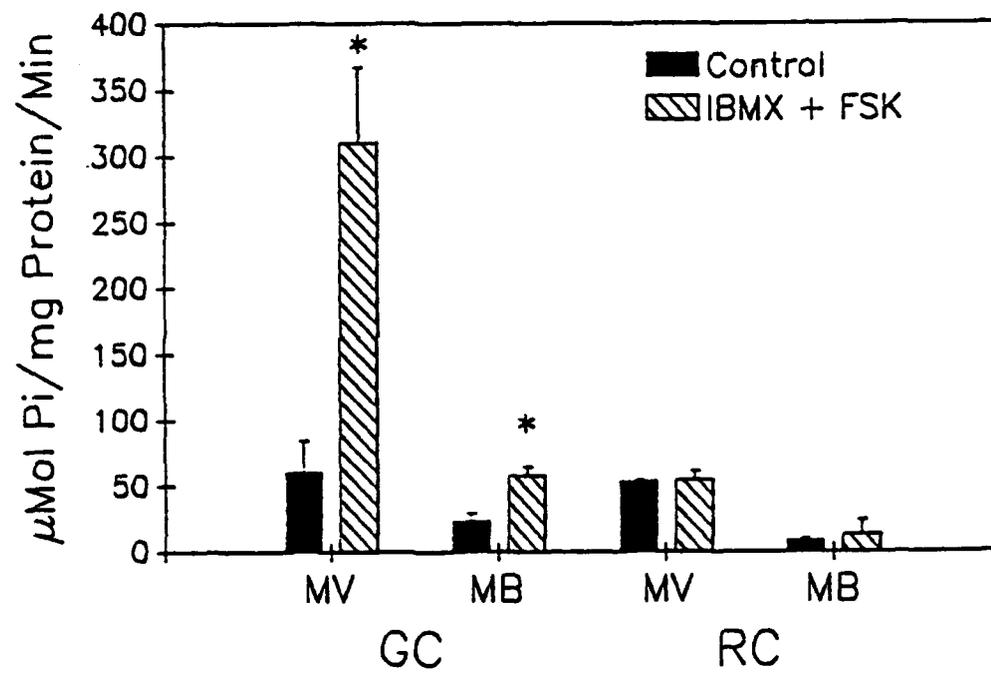


Figure 14:

The effect of bPTH (1-34) with IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

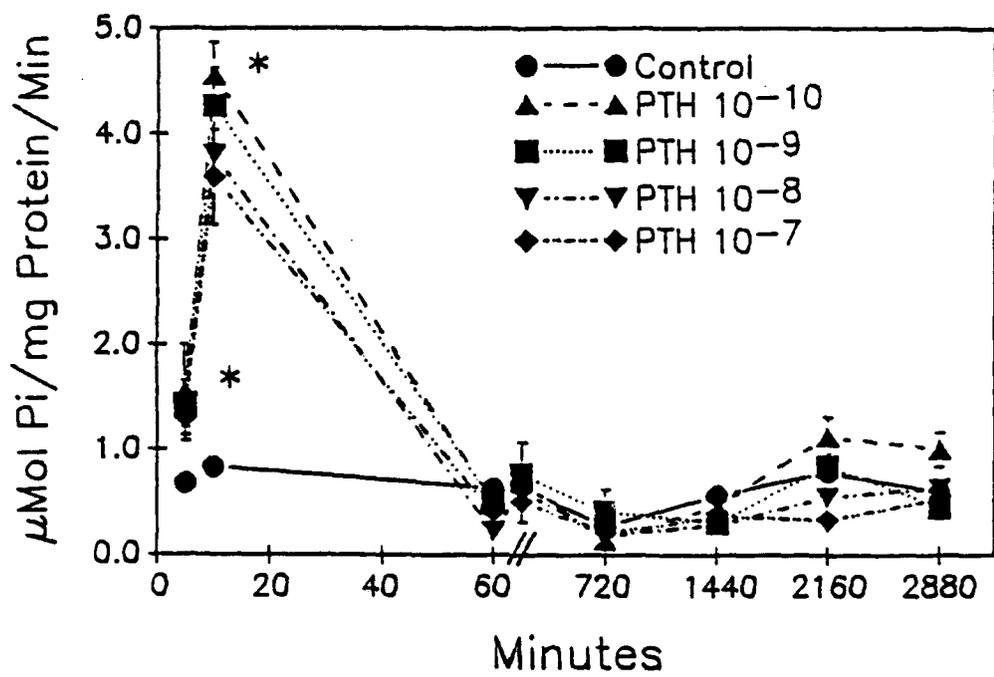


Figure 15:

The effect of bPTH (1-34) with IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

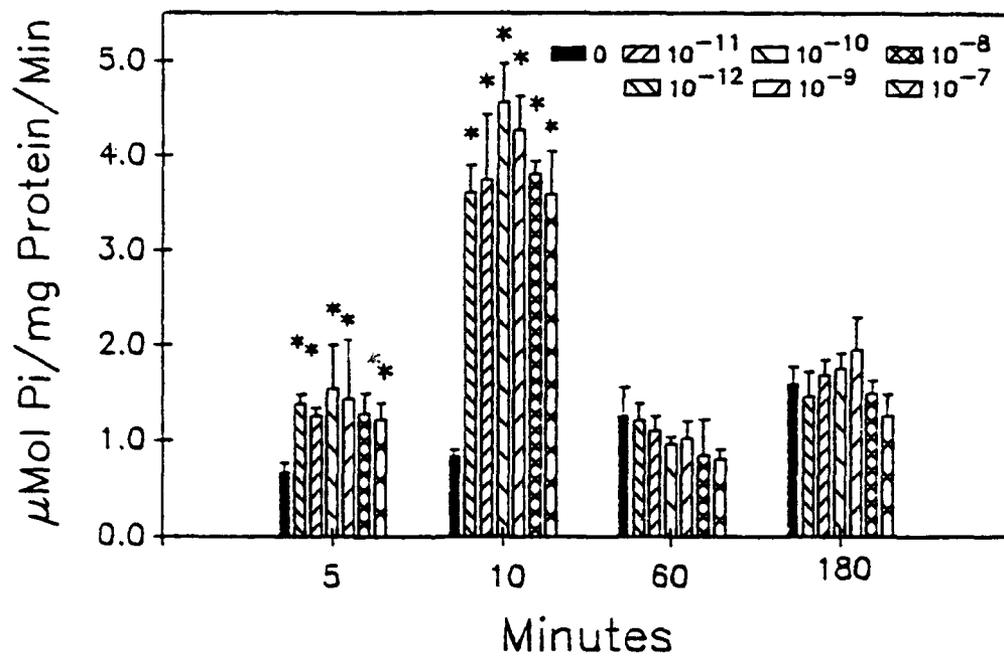


Figure 16:

The effect of bPTH (1-34) with IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral growth zone chondrocytes at 10 minutes.

Matrix vesicles and plasma membranes isolated from two T-75 flasks were combined for each sample. Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

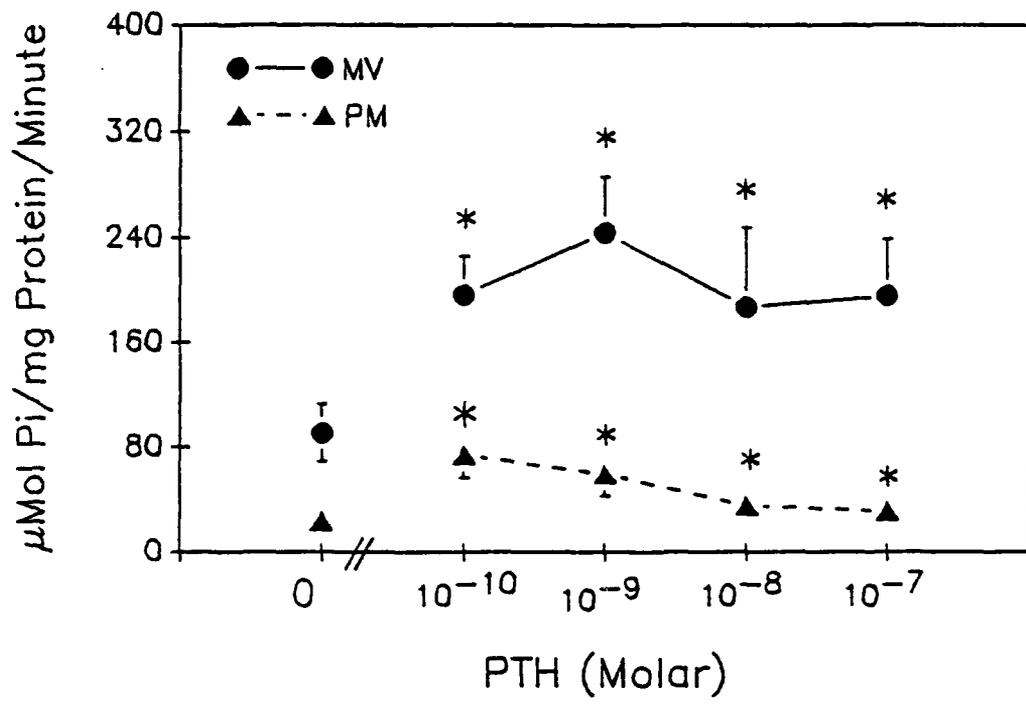


Figure 17:

The effect of bPTH (1-34) with IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral resting zone chondrocytes.

Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.

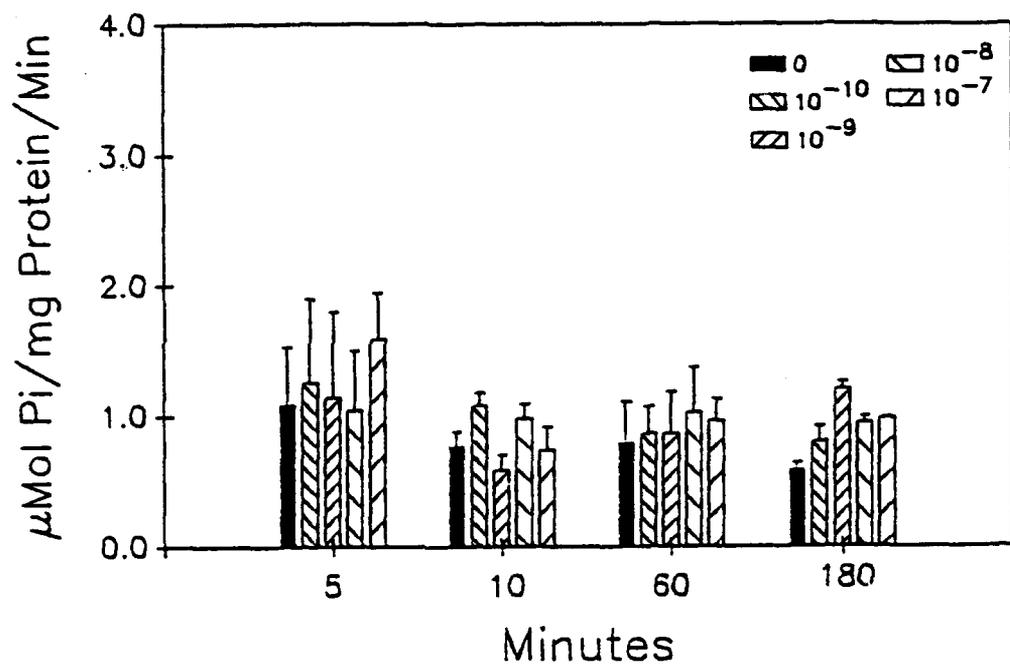
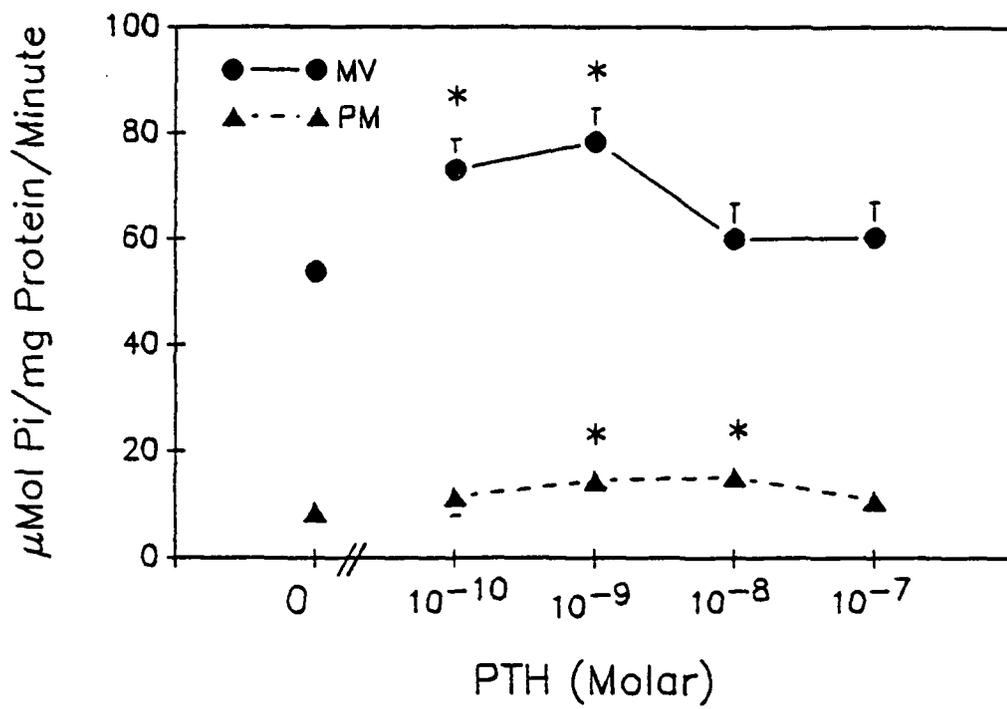


Figure 18:

The effect of bPTH (1-34) with IBMX and forskolin on the specific activity of alkaline phosphatase in confluent, fourth passage rat costochondral resting zone chondrocytes at 10 minutes.

Matrix vesicles and plasma membranes isolated from two T-75 flasks were combined for each sample. Data represent the mean \pm SEM of 6 samples. *p < 0.05 for sample vs control. Data shown are from a single experiment. Each experiment was repeated two times.



IV. DISCUSSION AND SUMMARY

Results from the present study confirm previous observations that cartilage is a target tissue for PTH (Takano et al. 1987; Levy et al. 1988; Pines and Hurwitz 1988; Livne et al. 1989). Enomoto et al. (1989) were the first to document the physicochemical and kinetic properties of PTH receptors on chondrocytes, demonstrating a single class of saturable, high affinity PTH binding sites on growth and resting zone cells of rabbit costal chondrocytes. They found growth cells exhibited more receptors and responded more strongly to the hormone than did resting cells, suggesting the expression of specific receptors and increases in the number of such receptors may play an important role in chondrocyte differentiation.

Thus, one may infer from the present data that PTH-induced stimulation of alkaline phosphatase (ALPase) activity not only depends on the state of chondrocyte maturation, but affects the degree of cartilage cell differentiation as well. This is particularly apparent from data demonstrating a biphasic, dose-dependent response to bPTH by cell layers from growth zone cartilage, as compared to the dose-dependent monophasic response exhibited by resting zone chondrocytes. These observations contradict previous studies demonstrating PTH-induced inhibition of mineralization-related phenotypes by growth plate chondrocytes. Chin et al. (1986) found physiologic levels of PTH stimulated cell division and suppressed cellular ALPase, indicating PTH caused chondrocytes to revert to a less differentiated state. They concluded that elevation in PTH

resulting from lowered circulating levels of Ca^{2+} inhibited mineral deposition in the growth plate and may act as a physiological protective mechanism to prevent a further drain on serum Ca^{2+} . Similarly, Kato et al. (1990) showed PTH caused 60-90% decreases in ALPase activity and matrix calcification in rabbit growth-plate chondrocyte cultures. Takano et al. (1987) propose that conflicting data between studies may be explained in terms of the unique characteristics of individual cartilage types *in vivo*, the particular PTH fragment(s) used, or the age of the chondrocyte population studied.

Numerous studies have demonstrated differential responses by chondrocytes to various fragments of PTH. Shurtz-Swirski et al. (1990) found human PTH (hPTH) 1-34 inhibited ALPase activity and stimulated mitogenesis in mandibular condylar cartilage *in vitro*. Conversely, hPTH (24-48) had no mitogenic effect but enhanced cartilage cell hypertrophy, while no significant effects on either mitogenicity or morphology were noted in hPTH (53-84)-treated cultures. Schluter et al. (1989) showed the hPTH fragments (1-34), (3-34) and (28-48) exerted a similar, dose-dependent, mitogenic effect in primary cell cultures of chicken chondrocytes. In contrast, hPTH fragments (43-68) and (52-84) had no effect. Using a series of synthetic hPTH peptides covering the central region of the hormone molecule (residues 25-47), the authors localized this mitogenic functional domain to a core region between amino acid residues 30 and 34.

Maturity of the chondrocyte population being studied has also been shown to influence responses to PTH. Koike et al. (1990)

demonstrated PTH (1-34) had a mitogenic effect on chondrocytes from embryonic chickens and fetal rabbits. In contrast, no mitogenic responses were observed in postnatal chick or rabbit chondrocytes. Interestingly, this age dependency was observed only with PTH, as fibroblast growth factor, epidermal growth factor, and insulin, stimulated chondrocyte proliferation irrespective of cartilage age. The authors concluded that PTH is a potent mitogen for embryonic chondrocytes, and that its mitogenic effect disappears selectively after birth. Conversely, PTH (1-84) has been shown to stimulate mitogenesis in chondrocytes from mouse mandibular condyles which had lost their proliferative activity with aging (Livne et al. 1989).

In addition to the factors mentioned thus far, it is evident that conflicting data among studies may in part be attributed to the unique properties of cartilage cells *in vivo*. Copray et al. (1988) observed prominent differences in the response to PTH by rat secondary mandibular condylar and primary costal cartilage in terms of matrix synthesis and cell proliferation. Because similar differences were not observed for a number of other factors examined, the authors concluded that most factors conducive to growth in primary cartilage also stimulate growth in condylar cartilage; however, exposure to growth substances that interfere with the differentiation of prechondroblasts into chondroblasts (e.g. PTH), a process that is specific for appositionally growing primary cartilage, may result in different responses between primary and secondary cartilages. Takano et al. (1987) studied the response of mandibular condylar cartilage,

nasal septal cartilage, and sphenoccipital synchondrosis to bPTH (1-34) and found increases in cAMP level, ornithine decarboxylase activity (a rate-limiting enzyme in polyamine biosynthesis), and glycosaminoglycan synthesis varied among all cartilage types. They concluded that such differences in the response patterns to PTH by distinct cartilage cell populations may reflect differences in the characteristics of these cells *in vivo*. Uchida et al. (1988) demonstrated that the long-term mechanical loading of chondrocytes may exert an alteration in their responsiveness to PTH. This is an especially interesting finding in consideration of the diverse mechanical forces placed upon costochondral cartilage as used in this study, compared to that from the epiphyseal growth plate of long bones.

The growth and maturation of cartilage, including stimulation of ALPase, is a time-dependent and orderly process that is regulated by a variety of hormones and metabolic factors. Several lines of evidence suggest cAMP may be one of the more important agents controlling these processes. Chin et al. (1986) found the inhibitory effects of PTH on ALPase activity in primary cultures of chicken epiphyseal growth plate chondrocytes were augmented by 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, and mimicked by the cAMP analogue N₆,O₂'-dibutyryl-adenosine 3',5'-cyclic-monophosphate, leading the authors to conclude that PTH was acting through the classic cAMP-mediated mechanism. Burch and Lebovitz (1981, 1983) used N₆-monobutyryl cyclic AMP to mimic the effects of PTH on cartilage growth *in vitro* and similarly concluded the hormone

triggered its effect by raising intracellular cAMP. Pines and Hurwitz (1988) used yet another agent, forskolin, to enhance cellular cAMP levels and mimic PTH-induced chondrocyte proliferation, again suggesting mediation via cAMP.

Results of this study indicate the increased ALPase response to bPTH in our cartilage model is not mediated by cAMP. This is particularly evident from the absence of a significant ALPase response when cellular cAMP levels were enhanced with IBMX and forskolin in resting zone chondrocytes. Although similar observations were not as obvious for growth zone cells, it is apparent PTH stimulates ALPase through a mediator other than cAMP when considering the lack of a biphasic ALPase response upon incubation of these cells in PTH with IBMX and forskolin. Kato et al. (1990) have made similar observations regarding chondrocyte proteoglycan synthesis, concluding that, while the nucleotides and PTH exerted similar effects, mediation occurred through different mechanisms. Somjen et al. (1990) have shown that rises in PTH-induced cellular cAMP may depend on the PTH fragment utilized. They demonstrated a mitogenic effect on rat epiphyseal cartilage cultures with concurrent increases in cellular cAMP for bPTH (1-84) and (1-34). While the bPTH (28-48) fragment showed similar mitogenic effects, it inhibited the increase in cAMP caused by bPTH (1-84). Interestingly, the authors proposed that because similar effects were observed in osteoblast-like cells (ROS 17/2.8), and because cAMP formation by PTH in these cells leads to bone resorption, that perhaps mid-region fragments of PTH might prove suitable for use *in vivo* to

induce bone formation without concomitant resorption. Thus, it is tempting to speculate that a dual receptor with distinct mediators (e.g. increased intracellular calcium and cAMP) similar to that described for bone is operating in our chondrocyte model. It may be that the interaction between these mediators is such that enhanced intracellular cAMP acts to inhibit the expression of pathways leading to increased intracellular calcium, thus inhibiting ALPase activity as observed in this study.

Despite the support for cAMP as an important mediator of PTH action in chondrocytes, previous studies suggest cAMP is not the sole secondary messenger of PTH-induced effects on cartilage cells. Somjen et al. (1990) found mitogenic-related effects on chondrocytes from rat epiphyseal cartilage were independent of increases in intracellular cAMP. In addition, studies failing to mimic certain PTH-induced effects on cartilage cells using agents enhancing cellular cAMP levels support the argument for additional mediators. For example, Schluter et al. (1989) found the effect of PTH on mitogenesis could not be mimicked by the addition of forskolin to primary cultures of chicken chondrocytes.

In summary, the effect of bPTH on chondrocyte ALPase specific activity appears to depend on the state of cell maturation. Furthermore, bPTH enhances differentiation of growth and resting zone chondrocytes by increasing the expression of an enzyme commonly associated with mineralization, alkaline phosphatase. To our knowledge, these results represent the first time that enhancement of mineralization related phenotypes has

been shown in a mature, primary, mammalian chondrocyte model. The fact that our results appear to conflict with physiologic mechanisms occurring in bone models is not entirely surprising. For example, the action of many anabolic hormones on bone is proposed to be mediated through local insulin-like growth factor-I (IGF-I) release. Elford and Lamberts (1990) have shown that while TGF beta-1 stimulates release of IGF-1 from osteoblasts, it is inhibitory in chondrocytes. They concluded that the regulation of local IGF-1 production in bone and cartilage may differ and illustrates the complex nature of local cytokine interactions in the two tissue types. While cAMP does not appear to mediate PTH-induced stimulation of ALPase in our study, previous reports suggest signal transduction may involve effects on calcium channels in a cAMP-independent manner (Schluter et al. 1989; Iannotti et al. 1990). This possibility warrants further study and is presently being investigated by this laboratory.

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VITA

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