MODULATION OF PTH MEDIATED SIGNAL TRANSDUCTION IN OSTEOBLASTS
BY FACTORS REGULATING CELLULAR GROWTH

by

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A thesis submitted in conformity with the requirements
for the degree of Masters of Science
Graduate Department of Pharmacology
University of Toronto

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ABSTRACT

Pertussis toxin (PTOX) treatment of ROS17/2.8 osteosarcoma cells has been reported to increase parathyroid hormone (PTH)–stimulated adenylyl cyclase (AC) and diminish PTH receptor desensitization. The objective of this study was to determine the effect of PTOX on AC activity in UMR106-01 osteosarcoma cells, and establish the mechanism of PTOX effects on this system.

UMR106-01 cells were shown to express Gi$_{1-2}$ and Go proteins and their inhibition by PTOX treatment of whole cells led to increases in both PTH and PGE$_2$ stimulated AC activity. This increased activity was not mediated by release of Gi inhibition of AC since acute inhibition of this protein in cell membranes did not alter AC activity. Therefore, other effects of PTOX on the cells were investigated and it was demonstrated that PTOX inhibits the growth of UMR 106–01 cells by inhibiting their response to their own secreted growth factors. Modulation of cellular growth by different media revealed that AC responsiveness of the UMR 106–01 cells is also increased under low growth conditions. These studies help to define the role of Gi/o proteins in osteoblasts and demonstrate that the responses of UMR 106–01 cells to two bone resorbing agents can be profoundly influenced by the local bone growth factors.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic-3,5'-adenosine monophosphate</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>CaR</td>
<td>calcium receptor</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DPM</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetracacetate</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>Forsk</td>
<td>forskolin</td>
</tr>
<tr>
<td>G protein</td>
<td>guanine-nucleotide binding regulatory protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>Gi</td>
<td>inhibitory guanine-nucleotide binding regulatory protein</td>
</tr>
<tr>
<td>Gq</td>
<td>guanine-nucleotide binding protein stimulatory to PLC</td>
</tr>
<tr>
<td>Gs</td>
<td>stimulatory guanine-nucleotide binding regulatory protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine 5'-3-O-(thio)triphosphate</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methyl-xanthine</td>
</tr>
<tr>
<td>IGFI</td>
<td>insulin-like growth factor I</td>
</tr>
<tr>
<td>IGFII</td>
<td>insulin-like growth factor II</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine diphosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NCS</td>
<td>newborn calf serum</td>
</tr>
<tr>
<td>NHE</td>
<td>sodium hydrogen exchanger</td>
</tr>
<tr>
<td>OAF</td>
<td>osteoclast activating factor</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet dervied growth factor</td>
</tr>
<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>PTOX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>rPTH(1–34)</td>
<td>rat parathyroid hormone (1–34)</td>
</tr>
<tr>
<td>s.d.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloracetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris [hydroxymethyl] aminomethane</td>
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1. INTRODUCTION

1.1. Overview of Extracellular Calcium Homeostasis

In 1883, Sidney Singer discovered the importance of calcium (Ca\(^{2+}\)), when he found that frog hearts could not beat without it. Calcium is involved in multiple biochemical and physiological processes in the body. It is required not only for muscle contraction but also for neurotransmitter release and blood coagulation. Calcium can also serve as an intracellular second messenger (Exton, 1987).

The concentration of Ca\(^{2+}\) present in the extracellular fluid (ECF) is in the order of \(10^{-3}\) M. This is a thousand fold higher than the amount of Ca\(^{2+}\) that is present intracellularly. Normally, Ca\(^{2+}\) concentrations in the ECF vary between 9–10 mg/dl (Guyton, 1992), therefore, it is apparent that serum calcium levels are very tightly controlled in the body (Borle, 1981; Rasmussen, 1986). Disturbances in serum calcium levels can lead to a multitude of detrimental effects. Hypercalcemia occurs when blood calcium levels rise above 12 mg/dl. Hypercalcemia can cause vomiting, constipation, confusion, lethargy and even coma. On the other hand, hypocalcemia occurs when serum calcium levels fall to 6.4 mg/dl, resulting in tetany, convulsions and even death, in cases of acute hypocalcemia. Therefore, it is essential for ECF calcium levels to be maintained within a narrow range (6.4–12 mg/dl).

Maintenance of calcium homeostasis is a complex process involving an intricate interplay of hormones, such as parathyroid hormone (PTH), vitamin D and calcitonin (Fig. 1). It involves several organ systems which act in concert to maintain normal Ca\(^{2+}\) levels. The bone, gut and kidney comprise the target organs acted on by the hormones responsible for maintaining Ca\(^{2+}\)
Figure. 1. Control of Calcium Homeostasis. Schematic representation of the major hormones and target tissues involved in calcium homeostasis (Favus MJ, ed, 1996).
homeostasis. Bone plays an important role in calcium homeostasis as it represents the largest reservoir of calcium in the body.

Decreases in serum calcium levels lead to the release of PTH from the parathyroid glands. PTH secretion initiates a cascade of events leading to the restoration of normal calcium levels, which in turn inhibits the release of PTH (see below for details).

In parathyroid cells, a rise in extracellular calcium leads to an increase in intracellular calcium and a decrease in intracellular cAMP, which in turn leads to the inhibition of PTH secretion. There are two mechanisms which allow parathyroid cells to respond to the changes in ECF calcium levels in this manner: 1) calcium-sensing receptor (CaR); and 2) plasma membrane calcium channels. Increases in extracellular calcium lead to the stimulation of CaR, which in turn activates at least two major signal transduction pathways, Gq and Gi. Stimulation of Gq leads to the production of inositol 1,4,5-trisphosphate (IP$_3$) which acts to release calcium from intracellular stores. This initial rise in intracellular calcium is accompanied by an influx of calcium via the plasma membrane calcium channels. Moreover, CaR activation also leads to stimulation of Gi which inhibits adenylyl cyclase (AC) leading to a decrease in intracellular cAMP levels. Together these effects of ECF calcium on parathyroid cells lead to a reduction in PTH secretion. The dynamics of PTH release from the parathyroids are much more complicated than presented here. For further details on this topic please refer to Pocotte et al.(1991) and Herbert and Brown (1995).

1.2. Parathyroid Hormone

PTH is a major bone resorptive agent. It is an 84 amino acid polypeptide with a molecular
weight of 9,500 daltons. Numerous structure function studies have been performed on PTH (Fig. 2). These studies reveal that the first 34 amino acids of this peptide can mediate the actions of the hormone on calcium homeostasis (Kronenberg, 1993). It is also known that the first 13 amino acids of the parathyroid hormone related–peptide (PTHrP), a factor produced by tumors that causes humorally mediated hypercalcemia, are identical to PTH. Blind et al. (1993), have shown that cAMP formation is equally stimulated by PTH (1–34) and PTHrP (1–34), in both rat bone and kidney cells.

The function(s) of the carboxyl–terminus of the PTH molecule remains elusive (Demay et al., 1985; Murray et al., 1989; Schulter et al., 1989). Some investigators have hypothesized that the C–terminus may play a role in the biosynthetic processing and secretion of the hormone (Lim et al., 1992; Orloff and Stewart, 1995).

1.2.1. Overview of the actions of PTH

The physiological actions of PTH are multiple and include: 1) increases calcium absorption from the gut; 2) increases calcium reabsorption and phosphate excretion from the kidney; and 3) increases calcium resorption from bone (see below for details). The intracellular actions of PTH are associated with the physiological actions of PTH. However, the exact link between the intracellular actions of PTH and its physiological actions still remain to be fully elucidated. These actions of PTH will be discussed in detail later. Briefly, the intracellular actions of PTH are as follows: 1) increases in cAMP levels (Chase and Aurbach, 1968; Rodan and Rodan, 1973; Hunt et al., 1976; Goltzman et al., 1976; Nissenson et al., 1979); 2) increases in inositol 1,4,5–trisphosphate and calcium levels (Farese et al., 1981; Meltzer et al., 1982;
Figure. 2. Stylized diagram to indicate distinct functional regions of PTH (panel A) and PTHrP (panel B). The shadings indicate the extent of the different functional domains of each protein (Mallette, 1991).
Rappaport et al., 1986; Hruska et al., 1987; Yamaguchi et al., 1987; Babich et al., 1989; Cosman et al., 1989); 3) activation of protein kinase A (Bringhurst et al., 1989); 4) activation of protein kinase C (Abou–Samra et al., 1989a); and 5) stimulation of the Na–H antiporter (Ganz et al., 1990).

1.2.2. Actions of PTH on the Kidneys and the Gut

PTH acts on the gut in an indirect manner, via stimulation of renal vitamin D₃ production. In the kidney, PTH stimulates 1–α hydroxylase to convert 25–OH–vitamin D₃ to its active metabolite 1,25–dihydroxycholecalciferol (Garabedian et al, 1972). 1α–25(OH)₂–vitamin D₃ then facilitates Ca²⁺ absorption from the gut, via active transport and facilitated diffusion (Mundy, 1990a). In general, PTH and vitamin D act synergistically to maintain serum calcium levels.

PTH also increases serum Ca²⁺ levels by stimulating calcium reabsorption in the kidney from the distal tubular nephrons. It also inhibits the proximal and distal renal tubular reabsorption of phosphate thereby increasing phosphate excretion in urine. Bicarbonate reabsorption from the proximal tubules is also inhibited by PTH. All of these actions of PTH are directed toward increasing serum calcium levels. Once that is achieved the circulating levels of PTH fall.

1.2.3. Actions of PTH on Bone

PTH has various complex and paradoxical effects on bone. Although it is a major bone resorptive agent, PTH displays both anabolic and catabolic activities. The catabolic actions of PTH are: 1) inhibition of procollagen synthesis; 2) stimulation of collagenase synthesis; 3) inhibition of alkaline phosphatase activity; 4) inhibition of osteocalcin synthesis; and 5)
promoting the release of osteoclast activating factors (OAFs).

The anabolic actions of PTH include an increase in osteoblast number following acute PTH treatment (Seyle, 1932; Jaffe, 1933; Shelling et al., 1933; Burrows, 1938; Canalis et al., 1989). It has been suggested that the anabolic effects of PTH may be mediated by the local growth factors produced by bone cells. For instance, Canalis et al. (1989), have shown that transient PTH treatment stimulates insulin–like growth factor I (IGFI) mediated collagen synthesis.

1.3. Site of PTH Action in Bone

Bone consists of two major cell types: osteoblasts that deposit bone and osteoclasts that resorb bone. The osteoblast is the target cell of PTH action. There seems to be a delicate equilibrium between the biological function of osteoblasts and osteoclasts, referred to as bone remodelling. This process is constantly occurring in the body, for the maintenance of skeletal and metabolic homeostasis. The skeletal need for bone remodelling arises through the course of the day as miniature fractures appear in the bone due to various mechanical stresses. These fractures are repaired via the bone remodelling process. The metabolic need for bone turnover is calcium homeostasis.

When the equilibrium between these two processes is disturbed, various disease states can arise. For instance, Paget's disease occurs when the equilibrium shifts in favor of bone deposition and osteoporosis occurs when the equilibrium shifts towards bone resorption.
1.3.1. Osteoblasts

Osteoblasts are complex cells derived from local mesenchymal stem cells. These precursor cells can differentiate into mature osteoblasts (Fig. 3) upon stimulation by growth factors (see review by Mohan and Baylink, 1991a; Baron, 1993). Mature osteoblasts produce a multitude of proteins, including Type I collagen and also non-collagen proteins, such as osteopontin and osteonectin (Table 1). In addition to these matrix proteins, osteoblasts also produce and secrete autologous growth factors such as insulin-like growth factors I (IGFI), insulin-like growth factor II (IGFII) and platelet derived growth factor (PDGF) (Table 2). These growth factors are stored in the bone matrix and can act as either autocrine (stimulating the osteoblasts from which they are released) or paracrine factors (stimulating nearby bone cells).

Osteoblastic cells express PTH receptors and are acted on by PTH (see below for mechanism). It has been demonstrated that PTH induces growth factor release from osteoblasts and that growth factors are able to modulate PTH responsiveness in osteoblastic cells. Canalis et al. (1989) showed that transient PTH treatments of fetal rat calvaria lead to a 2–4 fold increase in the concentrations of IGFI. Moreover, in human osteoblast-like SaOS-2 cells, IGFI has been demonstrated to inhibit PTH stimulated cAMP production (Goad and Tashjian, 1993). Thus, there seems to be a negative feedback loop in place, where PTH stimulates the release of growth factors which in turn act to attenuate the effects of PTH on osteoblasts.

Osteoblasts can also demonstrate differential expression of PTH receptor. For instance, Mitchell et al. (1990) have shown that the clonal osteosarcoma cell line UMR 106 exhibited three different morphological cell types that expressed different levels of PTH receptors. The small polyhedral cells, type A, had relatively few PTH receptors. The type B cells, cells with long
Figure. 3. Schematic of the osteoblast cell lineage. Progression of the mesenchymal stem cell through transitional stages, to osteoblast, osteocyte, and lining cell (Aubin and Kahn, 1996).
Table 1. Principal bone cell noncollagen products secreted to the bone matrix (Termine, 1993).

<table>
<thead>
<tr>
<th>Name</th>
<th>Approximate size</th>
<th>Potential function</th>
</tr>
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<tbody>
<tr>
<td>Thrombospondin</td>
<td>450,000 (trimer)</td>
<td>Cell attachment</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>440,000 (dimer)</td>
<td>Cell attachment, spreading Unknown</td>
</tr>
<tr>
<td>Biglycan (proteoglycan I)</td>
<td>170,000 (monomer)</td>
<td>Collagen Fibrillogenesis</td>
</tr>
<tr>
<td>Decorin (proteoglycan II)</td>
<td>120,000 (monomer)</td>
<td>Cell attachment, others unknown</td>
</tr>
<tr>
<td>Bone sialoprotein</td>
<td>75,000 (monomer)</td>
<td>Cell attachment, spreading Unknown</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>50,000 (monomer)</td>
<td>Ca²⁺, mineral binding; others unknown</td>
</tr>
<tr>
<td>Osteonectin</td>
<td>35,000 (monomer)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Matrix gla protein</td>
<td>9,000 (monomer)</td>
<td>Ca²⁺ binding, bone turnover</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>6,000 (monomer)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Growth factor activity produced by bone or isolated from bone matrix (Puzas, 1993).

- Transforming growth factor β 1,2, and 3
- Bone morphogenetic proteins 1 through 7
- Insulin–like growth factors I and II
- Platelet–derived growth factor
- Acidic and basic fibroblast growth factor
cytoplasmic extensions, express high levels of PTH receptors. Lastly, the type C cells, double circular cells with abundant cytoplasm, show less PTH receptors than type B. Since this is a clonal cell line they have proposed that this morphological heterogeneity reflects cells at different stages of the cell cycle.

1.3.2. Osteoclasts

Osteoclasts are bone resorbing cells derived from haematopoietic mononuclear cells in bone marrow (Fig 4). They are multinucleated cells with a ruffled border.

Osteoclasts are acted on by various systemic and local hormones (Table 3). They are indirectly stimulated by PTH to resorb bone (McSheehy and Chambers, 1986). This is mainly because osteoclasts do not express PTH receptors. PTH stimulates osteoblasts to release osteoclast activating factor (OAF) which leads to stimulation of osteoclastic breakdown of bone and release of calcium. The identity of OAF has not been established. Some investigators believe that OAF is not one molecule but rather a family of molecules (Mundy, 1990b). Interleukin-1 (a cytokine) has been shown to be released from both osteoblasts and activated monocytes. It is a potent stimulator of osteoclastic activity and it is believed to be one of the molecules that comprise OAF. Other cytokines known to stimulate osteoclasts are lymphotoxin and tumor necrosis factor. These factors are functionally related to interleukin-1.

More recent studies have shown that C-terminal fragments of PTH, such as PTH-(39–84) and PTH-(53–84), stimulate osteoclast precursor differentiation (Kaji et al., 1994). Therefore, the existence of a carboxyl-terminal PTH receptor has been proposed (see below for details). It has been suggested that these receptors are present not only on osteoblasts but also on osteoclast
Figure. 4. Osteoclast differentiation and life cycle. Osteoclast precursors are mononuclear pluripotent cells that differentiate along the osteoclast lineage. Monocyte-macrophage colony-stimulating factor (M-CSF) is an important cytokine required for survival of these precursors. As the osteoclast precursor cells become more differentiated they fuse to form immature osteoclasts. Systemic factors such as parathyroid hormone, 1,25-dihydroxyvitamin D₃, and interleukin-1 stimulate osteoclast precursor cell fusion. Mature osteoclasts have ruffled borders and are able to resorb bone upon appropriate stimulation. The osteoclast undergoes apoptosis (programmed cell death) during bone remodelling and in response to agents such as transforming growth factor-β (TGF-β), estrogens, and bisphosphonates (Mundy, 1996).
Table 3. List of factors that regulate osteoclastic activity (Mundy, 1996).

<table>
<thead>
<tr>
<th>Systemic Hormones</th>
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<tbody>
<tr>
<td>PTH</td>
</tr>
<tr>
<td>1,25–dihydroxyvitamin D₃</td>
</tr>
<tr>
<td>Calcitonin</td>
</tr>
<tr>
<td>Local Hormones</td>
</tr>
<tr>
<td>Interleukin–1</td>
</tr>
<tr>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>Colony–Stimulating Factor–1</td>
</tr>
<tr>
<td>Osteoclastpoietic Factor</td>
</tr>
<tr>
<td>Interleukin–6</td>
</tr>
<tr>
<td>Gamma Interferon</td>
</tr>
<tr>
<td>Transforming Growth Factor–β</td>
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<tr>
<td>Other Factors</td>
</tr>
<tr>
<td>Retinoids</td>
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<tr>
<td>Transforming Growth Factor–α</td>
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<td>Gallium Nitrate</td>
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</tbody>
</table>
1.4. Mechanism of action of PTH

1.4.1. The PTH/PTHrP Receptor

Both PTH and PTHrP are thought to act via the PTH/PTHrP receptor (PTHR). The receptor belongs to the superfamily of seven transmembrane domain receptors (Fig. 5), and shares close homology with the calcitonin and secretin receptors (Abou-Samra et al., 1992).

The PTHR has been found in both bone and kidney cells and the results of Orloff et al. (1992), suggest that PTH receptors in human bone and kidney cells are very similar, if not identical. The PTHR has been cloned from both rat bone and opossum kidney cells (Juppner et al., 1991; Abou–Samra et al., 1992). There is 78% amino acid identity between these two receptors. The PTHR seems to be well conserved amongst mammalian species.

Until recently, only one form of the receptor was known, however, two novel PTH receptors have now been characterized. Usdin et al. (1995) have identified the PTH2 receptor, which is responsive to PTH(1–34) but not to PTHrP(1–36). The distribution of the PTH2 receptor is restricted to only brain and pancreas, unlike the PTH/PTHrP receptor which has a more widespread tissue distribution. Although bone is one of the major sites of PTH action, the PTH2 receptor does not seem to be expressed in bone cells. It has been shown that the human osteosarcoma cell lines, Saos2 and G–292, do not express detectable mRNA for the PTH2 receptor. Therefore, the physiological importance for this receptor is unclear.

In addition, another PTH receptor has also been identified. This novel receptor has specificity for the carboxyl–terminus of PTH (Inomata et al., 1995). This carboxyl–terminus
Figure. 5. Schematic representation of the rat PTH/PTHrP receptor. EC = extracellular; IC = intracellular (LeRoith et al., 1994).
receptor for PTH (C–PTHR) was found on rat osteoblastic cells (ROS17/2.8) and rat parathyroid cells (PT–r3). Other clonal cell lines tested showed either low (UMR 106, opossum kidney) or no binding (YCC, SaOS–2, LLCPK₁, and MG63) to the to C-terminal PTH fragments. The biological significance of this receptor still remains to be elucidated and the second messengers involved in mediating its actions are also unknown. Therefore descriptions in the following sections only apply to the PTH/PTHrP receptor.

1.4.2. PTH Receptor Signal Transduction: Overview

The PTH receptor activates two major signal transduction pathways, which generate various second messengers. PTHR activates Gs (stimulatory guanine–nucleotide protein), which stimulates adenylyl cyclase (AC) leading to an increase in cyclic AMP levels (Chase and Aurbach, 1968; Rodan and Rodan, 1973; Hunt et al, 1976; Goltzman et al, 1976; Nissenson et al, 1979). PTHR also activates Gq which stimulates phospholipase C to activate the protein kinase C pathway (Abou–Samra et al, 1989a; Mitchell et al., 1995).

1.4.3. Second Messengers

Cyclic–AMP (cAMP)

PTH stimulation causes a rise in the levels of intracellular cAMP. This in turn leads to the activation of cAMP–dependent protein kinase A (PKA) which phosphorylates various protein substrates. cAMP has been identified as one of the major second messengers involved in mediating PTH signal transduction, both in kidney and bone. Evidence for a role of cAMP in the actions of PTH has come from various studies done by Chase and Aurbach (1967; 1968), which
have shown that agents which increase cAMP levels also increase calcium transport in the kidney. Other investigators have also reported similar observations of increased renal cAMP production upon PTH stimulation (Michelakis, 1970 and Steiner et al.; 1972). Further evidence of an involvement of cAMP in PTH signal transduction has come from studies demonstrating the ability of dibutyryl cAMP, a poorly hydrolyzable analogue of cAMP, to mimic the actions of PTH (Rasmussen et al., 1968; Russell et al., 1968).

The importance of cAMP as a mediator of the actions of PTH has also been shown in bone cells (Rodan and Rodan, 1973; Peck et al., 1973; Majeska et al., 1978; Forrest et al., 1985). For instance, Dietrich et al. (1976) showed that bone collagen synthesis was inhibited by PTH and they proposed that this effect was mediated by cAMP. Moreover, the inhibition of alkaline phosphatase activity has also been postulated to be mediated by cAMP (Majeska and Rodan, 1982). Increases in the levels of cAMP have been shown to increase collagenase production (Civitelli et al., 1989). All of these actions of PTH are catabolic in nature, therefore it is difficult to imagine that cAMP may be involved in mediating the anabolic actions of PTH.

Nonetheless, depending on the cell type, increases in the levels of cAMP have been associated with both stimulation and inhibition of cell proliferation. cAMP mediated inhibition of cell proliferation has been demonstrated in smooth muscle and mesangial cells. In Swiss 3T3 cells, cAMP was shown to stimulate cell growth. In bone cells, insulin–like growth factor I (IGFI) is believed to mediate the anabolic actions of PTH. Transient treatment with PTH leads to increases in the local production of IGFI, however, it is not known whether this is mediated by cAMP (Canalis et al., 1989).

In addition, it has been shown that although cAMP is a major mediator of the action of
PTH, full hormonal effect was not seen in the presence of 8BrcAMP (an analog of cAMP) alone. Civitelli et al. (1989) demonstrated that the Ca\(^{2+}\)/PKC system plays a role in mediating some of the actions of PTH. Therefore, it is apparent that the full physiological actions of PTH are mediated by the interaction of several signal transduction systems.

**Calcium**

PTH stimulation causes increases in intracellular levels of calcium. This occurs by two processes: a) activation of the PLC pathway; and b) activation of calcium channels.

**PLC pathway**

PTH-mediated stimulation of PLC leads to the hydrolysis of membrane phospholipids, mainly phosphoinositol-4,5 bisphosphate (PIP\(_2\)), to generate inositol-1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG) (Hruska et al., 1987). These second messengers then act on different effectors. IP\(_3\) induces the release of calcium from intracellular stores and DAG acts to stimulate protein kinase C (PKC). Thus, PTH activation not only causes an increase in calcium levels but also causes translocation and activation of PKC at the membrane (Abou-Samra et al., 19889a; Fujimori et al., 1992; Friedman et al., 1996).

The mechanism of activation of phospholipid metabolism by the PTHR has not been well characterized. There are several potential mechanisms by which receptors activate phospholipid turnover, depending on the subtypes of PLC expressed in the cell. For example, G protein coupled receptors activate the PLC \(\beta\) family of proteins (PLC\(\beta\) 1–4) predominantly via G proteins of the Gq/11 family. PLC \(\beta2\) can also be stimulated by G protein \(\beta\gamma\) subunits (Camps
et al., 1992; Smrcka and Sternweis, 1993) and it has been suggested that this is the mechanism by which receptors coupled to PTOX sensitive G proteins stimulate PLC. Recent studies in osteoblastic UMR 106 cells have demonstrated PLC activation by PTHR coupling to Gq/11 proteins (Mitchell et al., 1995). It remains to be seen if this is also the mechanism of PLC activation in other cell types expressing PTHR.

PLC activity in cells can also be mediated by growth factor tyrosine kinase type receptors which stimulate PLC γ subtypes. PLC γ is associated with the mitogen activated protein kinase (MAPK) pathway, one of the major growth stimulation pathways in cells. The MAPK pathway is activated by both tyrosine kinase type receptors, as well as G protein coupled receptors. Both cAMP and βγ subunits of heterotrimeric G proteins have also been shown to stimulate the MAPK pathway (Faure et al., 1994). It is not clear how PTH activates cellular growth in bone cells. It is possible that this occurs as a result of PLC β activation. Alternatively, PTH stimulation of the release of osteoblastic growth factors that can activate PLC γ may be responsible. Clearly more work needs to be done to understand the anabolic actions of PTH.

**Calcium Channels**

Yamaguchi et al. (1987) have shown that PTH stimulates calcium channels in UMR 106 cells. Their studies showed that PTH stimulates the activation of two different types of Ca\(^{2+}\) channels; cAMP-independent and cAMP-dependent Ca\(^{2+}\) channels. The cAMP-independent channel allows rapid increases in intracellular Ca\(^{2+}\) and is rapidly inactivated. On the other hand, the activation of the cAMP-dependent channel leads to a slow rise in intracellular Ca\(^{2+}\) levels.

Furthermore, Bizzarri and Civitelli (1994) have suggested that the PTH receptor couples
to a Ca$^{2+}$ channel via a PTOX–sensitive G protein, in a cell cycle dependent manner.

**Na–H antiporters**

PTH has been shown to affect cytosolic pH. There is evidence to suggest that changes in pH may play an essential or permissive role in promoting cellular proliferation (Grinstein et al., 1989). For instance, Reid et al. (1988) have shown that, in osteoblasts, alkalization of cellular pH parallels increases in [$^{3}$H]thymidine incorporation. Other evidence for this notion is as follows: 1) most mitogens activate the Na–H exchanger; 2) cytoplasmic alkalization can induce proliferation in the absence of mitogens; and 3) cell growth is blocked by inhibitors of Na–H exchange (Grinstein et al., 1989).

The mechanism through which PTH activates the Na–H exchanger is not known. Four isoforms of the Na–H exchanger have been identified and they are termed NHE-1 to NHE-4. Due to the conflicting reports of Na–H regulation, it is difficult to assess which signalling mechanism is important for Na–H modulation. Arzani et al. (1995a) have reported that osteoblasts (UMR 106) express only NHE-1, and that PTH and PTHrP activate NHE-1 in a cAMP–dependent manner. These same investigators have also shown that NHE-3 is the only isoform of the Na–H exchanger present in opossum kidney cells where it can be inhibited by both the PKA and PKC pathways (Arzani et al., 1995b). However, other studies have shown that NHE-1 activation via β–adrenergic receptors is Gs independent (Barber et al., 1989; 1992; Barber and Ganz, 1992). In fact, Voyno–Yasenetskaya et al. (1994) have reported that Go13 regulates the NHE-1 antiporter. Moreover, Dhanasekaran et al. (1994) have reported that both Go12 and Go13 are able to activate the antiporter.
It seems that there are proponents for both cAMP–dependent and cAMP–independent regulation of the antiporter. Whether PTH regulates the antiporter in a cAMP–independent manner remains to be seen. It is possible that the PTH receptor may activate the antiporter via both cAMP–dependent and cAMP–independent mechanisms.

1.5. Modulation of the Adenylyl Cyclase Pathway

PTH–mediated activation of adenylyl cyclase is clearly an important mechanism of regulating its target cell function. Therefore modulation of this pathway in PTH target cells is likely to have profound effects on the physiological actions of PTH.

Adenylyl cyclases are membrane bound enzymes that convert ATP to cAMP, which in turn leads to the activation of cAMP–dependent protein kinases. Eight types of AC are known to exist (Fig. 6). Although Gs is the primary stimulator of all known adenylate cyclases, these enzymes can be modulated by other factors. Type I, Type III and Type VIII AC are sensitive to calcium/calmodulin activation (Taussig and Gilman, 1995). Type II and Type IV AC are insensitive to calcium/calmodulin but are conditionally stimulated by G protein βγ subunits. It has been proposed that for this βγ regulation to occur, the necessary concentrations of βγ must be contributed by other G proteins in addition to Gs, such as Gi and Go type proteins (Tang and Gilman, 1992). Furthermore, Type V and Type VI AC have been shown to be sensitive to inhibition by Gi1, Gi2 and Gi3 and low concentrations of Ca²⁺. Type I adenylyl cyclase is also inhibited by the α–subunits of these three Gi proteins, as well as Go. Type II and IV AC are insensitive to Gi/Go regulation (Taussig et al., 1994).

Modulation of adenylyl cyclase response in PTH target cells has been studied by a number
Figure. 6. Patterns of regulation of adenylyl cyclase activity. PKC, protein kinase C; CAM, calmodulin; AC, adenylyl cyclase (Taussig and Gilman, 1995).
of investigators. Pines et al. (1986) have shown that, in rat osteosarcoma (ROS) cells, pertussis toxin treatment causes increases in adenylyl cyclase responsiveness to both PTH and isoproterenol. Bordetella pertussis toxin or islet-activating protein catalyzes the NAD*-dependent ADP-ribosylation of Gi/Go type proteins which stabilizes the heterotrimeric form of these G proteins and renders them inactive. Thus, Pines et al. (1986) found that knocking out PTOX sensitive substrates in ROS cells leads to an increase in agonist stimulated cAMP accumulation without affecting the basal cAMP production. They offered two potential explanations for their observations: 1) tonic inhibition of adenylyl cyclase by Gi; and 2) PTH mediated activation of Gi. They suggested that relief of tonic inhibition upon PTOX treatment does not fully explain their data, since they did not observe an increase in the basal levels of cAMP.

Their proposal of dual coupling of the receptor, to both Gs and Gi, is intriguing. It is not obvious why the PTH receptor would mediate both the stimulation (Gs) and the inhibition (Gi) of the adenylyl cyclase system, and the biological importance of such opposing actions mediated by the receptor remains unclear. More work needs to be done to clarify this issue.

Work done by Abou-Samra et al. (1989b) have suggested that PTOX treatment of ROS cells leads to an increased expression of the receptor and attenuates the agonist-induced cAMP-dependent downregulation of PTH receptors. They concluded that PTH receptor expression in ROS cells is tonically inhibited by PTOX-sensitive substrates.

It is apparent that the actions of PTOX on osteoblastic cells are complex. Further investigations need to be carried out to understand the role of Gi/Go type proteins in these cells.
1.6. Gi/Go Modulate Cellular Growth in Some Cell Types

There is evidence to suggest that Gi/Go type proteins may be involved in mediating cell growth. In 1987, Murayama and Ui, showed that PTOX treatment of Swiss 3T3 cells led to an inhibition of cell growth. In 1991, LaMorte et al. showed that microinjection of G\(\alpha_i\) antibodies in mouse Balb/c3T3 fibroblasts resulted in an inhibition of DNA synthesis. Furthermore, Moxham et al., in 1993, using a tissue-specific G\(\alpha_i\) knock out model, showed that G\(\alpha_i\) was essential for the neonatal growth of these tissues (fat, muscle and liver).

Gi/Go type proteins have also been implicated in aberrant growth. Mutations in the G\(\alpha_i\) gene (gip2) have been found in both adrenal and ovarian tumors (Lyons et al., 1990). It has also been demonstrated that the gip2 oncogene induces neoplastic transformation of some, but not all cells into which it was transfected (Pace et al., 1991; Gupta et al., 1992). Therefore, it has been suggested that the gip2 mutation alone is not enough to promote neoplastic transformation and other tissue-specific factors may influence the transformation potential of the gip2 mutation.

This cumulative evidence suggests that PTOX substrates are important in cellular growth regulation at least in some cell types. The effect of PTOX treatment on the growth of osteoblastic cells, however, has not been addressed.

1.7. Objectives

Osteoblasts play a key role in bone remodelling; they are directly responsible for bone deposition and indirectly responsible for bone resorption. Therefore, it is important to understand both the mechanisms underlying growth and maturation of these cells, as well as factors that can modulate osteoblast responsiveness to the systemic and local hormones and growth factors that
regulate osteoblastic activity. Determining the basic mechanisms of these processes may help in the development of better drug treatment regimens for bone degenerative diseases, such as, osteoporosis. Since previous studies have indicated that PTOX can modulate PTH actions in bone cells the objective of this study was to determine the role of pertussis toxin–sensitive G proteins (Gi/Go) in regulating osteoblastic function and cellular growth.

The specific aims were as follows:

1) To establish the effect of PTOX treatment on PTH activation of adenylyl cyclase in UMR 106–01 osteoblasts.

2) To determine if the PTHR is coupled to Gi/Go type proteins.

If the PTHR is not coupled to Gi/Go type proteins then:

3) To determine the mechanism by which PTOX affects adenylyl cyclase response to PTH.
2. MATERIALS AND METHODS

2.1. Materials

Anti-Gso and anti-Gia were purchased from Upstate Biotechnology. Anti-Goo was a generous gift from Dr. J.K. Northup, National Institute of Mental Health, Bethesda, MD. Tissue culture reagents (antibiotic antimycotic solution, Dulbecco's Modified Eagle Medium and Dulbecco's Modified Eagle Medium: Hams F12) were all purchased from Gibco BRL. Growth factors; EGF, IGFI and IGFII, were obtained from Boehringer Mannheim. Pertussis toxin was purchased from ICN or Sigma. All radiochemicals were purchased from DuPont NEN ([3H] Adenine, [3H] Thymidine, [3H] cAMP, [14C] cAMP, [32P] ATP and [32P] NAD).

2.2. Cell Culture and Treatments

UMR 106-01 osteosarcoma cells were kindly provided by Dr. J. Aubin at the University of Toronto and by Dr. N.C. Partridge at St. Louis University. These cells have been widely used as a model to study osteoblast function. The cells used in these studies ranged from passage# 18–30. The cells were grown in either 50:50 Dulbecco's Modified Eagle Medium (DMEM) and Hams F-12 supplemented with 5% fetal calf serum (high growth medium) or DMEM supplemented with 10% newborn calf serum (low growth medium). The medium also contained 100 units/ml penicillin, 100 ug/ml streptomycin and 0.25 ug/ml amphotericin B. The cells were kept at 37°C in 5% CO2 in air, subcultured once a week and the medium was changed every 2–3 days.

The cells were grown for 2–3 days prior to pertussis toxin (PTOX) treatment. On day 4,
the cells were serum deprived in the presence and absence of PTOX (50 ng/ml) for 39-48 hours. Serum was added to some cell populations 12-15 hours before the assay for [³H] thymidine incorporation. PTOX was also readded at that time.

2.3. Adenylyl Cyclase Assay

2.3.1. Whole Cell Adenylyl Cyclase Assay

UMR 106-01 cells were plated at 5x10⁴ cells/well (for high growth cells) and 1x10⁵ cells/well (for low growth cells) in 24 well plates and grown for 2-3 days. The assay used was similar to that of Demay et al. (1985) with some modifications. Prior to the assay the cells were incubated with 1 uCi/ml [³H] adenine for 2 hours at 37⁰ C. Cells were incubated in the absence and presence of stimulators (PTH, Forskolin, Prostaglandin E₂) in the presence of 0.2 mM isobutylmethylxanthine for 20 minutes at room temperature. At the end of the incubation intracellular cAMP was extracted with 10% trichloracetic acid precipitation. Then samples supplemented with a known amount of ¹⁴C cAMP as a tracer (3000 cpm), were passed over Dowex and Alumina columns to separate cAMP from other labelled nucleotides and counted in a β scintillation counter (Salomon et al., 1974).

2.3.2. Membrane Adenylyl Cyclase Assay

Membranes were prepared from cells grown in flasks (75cm²) which had undergone the same treatment as cells in the whole cell assay. The cells were plated at 3.75x10⁶ cells/flask (for low growth cells) and 1x10⁶ cells/flask (for high growth cells), trypsinized (0.1% trypsin, 0.58 mM EDTA in Earle's balance salt solution) and counted using a hemocytometer. The cells were
washed several times in serum-containing medium and then in phosphate buffered saline. A P2 pellet was prepared by suspending cells in hypotonic solution (50 mM Tris pH 8.0, 2 mM MgCl₂, 1 mM DTT) and disrupted using a polytron homogenizer. The homogenate was centrifuged at 500 rpm for 5 min (Beckman GPR centrifuge) to remove unbroken cells and nuclei, the supernatant was collected and centrifuged using a Beckman Model J2-21M Induction Drive Centrifuge, at 14,000 rpm for 30 min at 4°C. The resulting P2 pellet was resuspended in hypotonic solution containing 12% sucrose and frozen at −70°C until assay.

In some assays membranes were pretreated with pertussis toxin before adenylyl cyclase assay. PTOX was pre-activated by incubation at 37°C for 10 min in the presence of 10 mM DTT. Membranes were incubated with 25 µg/ml of activated PTOX for 10 minutes at 37°C in 1 mM ATP, 10 mM thymidine, 0.2 mM GTP and 1 mM NAD. Control membranes were incubated without PTOX, in the presence of all other components of the ADP-ribosylation assay. The total reaction volume was 100 ul. The reaction was stopped by the addition of 1 ml ice cold buffer (25 mM Tris pH 7.5). The membranes were then spun at 14,000 rpm in a microfuge for 5 minutes. After two more washes in buffer the membranes were resuspended in hypotonic solution containing 12% sucrose.

Adenylyl cyclase assays were performed in a final assay volume of 100 ul. The assay buffer contained 30 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM DTT, 100 uM ATP (containing 500,000 cpm [³²P] ATP), 0.5 mM IBMX, 0.01 M phosphocreatine and 0.3 mg/ml creatine phosphokinase. The stimulators (PTH, PGE₂, GTPγS, Forskolin) or vehicle were added to the assay buffer prior to membrane addition. At the start of the assay, 25–50 µg of protein was added to each tube and incubated at 30°C for 30 minutes. The reaction was stopped as described in
Mitchell and Goltzman (1990) and \(^{32}\)PcAMP was isolated by the method of Salomon et al. (1974).

2.4. ADP–Ribosylations

UMR 106–01 membranes were ADP–ribosylated with PTOX in the presence of \(^{32}\)P NAD. The total reaction volume was 60 ul. The assay was carried out as described by Ribeiro–Neto et al. (1985). The reaction buffer consisted of 20 mM ATP, 1 mM GDP, 0.1 mM EDTA, 10 mM Tris pH 7.5, 50 mM thymidine and 0.02 mg/ml BSA. At the start of the assay, 10 ug of protein was added to each tube and incubated at 37ºC for 30 min. The reaction was stopped with ice cold 20% TCA and the samples were kept on ice for 20 min. Then the pellets were washed in ethyl ether at least twice. After the ether had evaporated, the samples were taken up into sample buffer (0.0625 M Tris pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol containing bromophenol blue) and run on an SDS–PAGE gel. The gel was fixed in 40% methanol overnight, rehydrated, dried and prepared for autoradiography.

2.5. Thymidine Assay

UMR 106–01 cells were plated at 1x10^4 cells/well in 24–well plates and grown for two days. On day three the cells were serum deprived and treated with or without 50 ng/ml pertussis toxin (PTOX) for 24 hours at 37ºC. On day four stimulators were added and the cells were cultured further for 15–21 hours. Cells are incubated with 1 uCi/ml \(^{3}\)H] Thymidine for 1 hour prior to the assay. Cells were rinsed 1–2 times with 0.5 ml phosphate buffered saline, precipitated with 10% trichloracetic acid and digested with 0.5 N NaOH. An aliquot of the NaOH–
solubilized sample was used for β scintillation counting and protein determinations were made using Lowry protein assay.

2.6. Protein Assays

2.6.1. Relative Protein Assessment

To normalize assay results for differences in cell number in each well, the relative protein present was determined. Amido black dye (0.5 ml) was added to each well for 5 min to stain trichloracetic acid precipitated cellular protein. Each well was rinsed 2 times with water and 0.5 ml elution solution (0.025 N NaOH, 50 uM EDTA, 50% EtOH) was added to each well to solubilize the proteins. The optical density (OD) of each sample was read on a spectrophotometer (Beckman DU-65) at a wavelength of 630 nm.

In some experiments cell numbers were calculated based on this method by comparing OD measurements with a standard curve generated using known numbers of cells.

2.6.2. Lowry Protein Assay

Standard protocol was used (Lowry, 1951) with minor changes. Briefly, the samples were made up to a total volume of 200 ul. The Lowry C solution consisted of 2% Na₂CO₃, 0.5% CuSO₄ and 1% NaK tartarate in a ratio of 50:1:1. The Lowry E solution consisted of 2N folin:H₂O in a ratio of 1:1.5. The optical density of each sample was read on a spectrophotometer (Beckman DU-65) at a wavelength of 750 nm.
2.6.3. Amido Black Protein Assay

The assay was performed as described by Schaffner and Weissmann (1973) with minor modifications. The samples were made up to a volume of 200 ul and precipitated by addition of 50 ul of 90% TCA. Precipitates were immobilized on nitrocellulose, stained with amido black and eluted into elution solution and optical density was measured, as outlined above.

2.7. SDS–PAGE and Western Blotting

Membrane samples were run on 11% polyacrylamide gels by the method of Laemmli (1970). The running buffer consisted of 25 mM Tris, 190 mM glycine and 0.1% SDS. Proteins were electrophoretically transferred onto nitrocellulose in a transfer buffer containing 25 mM Tris, 190 mM glycine and 20% methanol. Membranes were probed for the following G protein α-subunits: Gs, Gi and Go using specific primary anti-sera and then visualized with an anti-rabbit IgG, horseradish peroxidase linked whole antibody followed by exposure to ECL substrate solution.

2.8. Data Presentation

The data are reported as mean ± standard deviation of triplicate determinations within the same experiment, unless otherwise specified. Figures illustrate a representative experiment and results obtained from similar experiments are listed in tables. One tailed student's t test was used to determine significant increases of treatment groups over controls in all experiments except those involving conditioned medium, in which case a two tailed t test was used.
3. RESULTS

3.1. Effect of PTOX Treatment

The effect of treating UMR 106-01 cells with PTOX for 24-48 hours in culture is shown in Fig. 7 and Table 4. Similar to previous reports in ROS cells, the results show that PTOX treatment of UMR 106-01 cells leads to an increase in adenylyl cyclase responsiveness. The cells were treated with or without PTOX in serum-containing medium or serum free medium. PTH stimulation was seen under all treatment conditions, however, the magnitude of the stimulation varied considerably. The differences seen in AC responsiveness between cells grown in 5% FCS with and without PTOX were not statistically significant, as determined by the student's t-test. Compared to vehicle pretreatment PTOX pretreatment of cells grown in serum-free medium showed at least a two fold increase in PTH-stimulated AC activity (Fig. 7b; Table 4). The PTOX induced differences in AC responsiveness were statistically significant in the serum free cells. The cells treated with PTOX under serum free condition were the most responsive to PTH while the lowest PTH stimulation was seen in the group of cells that were treated with serum in the absence of PTOX.

3.2. Generality of the PTOX Effect

PGE₂ was used to establish if similar increases in AC responsiveness in UMR 106-01 cells could be seen with agents other than PTH (Fig.8). The experiments were carried out under serum-free conditions since the greatest effect of PTOX was obtained under these conditions. PGE₂ stimulated AC activity in a dose-dependent manner. PTOX treatment augmented PGE₂ stimulated AC activity. Thus the effect of PTOX on AC responsiveness did not appear to be
Figure. 7. Effect of PTOX on PTH stimulated adenylyl cyclase activity in whole cells. The UMR 106-01 cells were grown in 24-well plates in a) 5% FCS containing medium with or without pertussis toxin (50 ng/ml); and b) in serum free medium with or without pertussis toxin (50 ng/ml). The increases in AC responsiveness seen under serum containing conditions were not statistically significant (p> 0.05 at 10^{-7} M PTH), however, the differences seen under serum free conditions were statistically significant (p< 0.005 at 10^{-7} M PTH). The values represent the mean and s.d. of triplicate determinations within this experiment. The protein values were determined to be similar between PTOX treated and untreated cells, using Lowry protein assay.
Table 4. Effect of PTOX on PTH stimulated adenylyl cyclase activity (see Fig. 7 for more details).

<table>
<thead>
<tr>
<th>Expt#</th>
<th>Treatment condition</th>
<th>Fold Stimulation (10 nM PTH)</th>
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<tbody>
<tr>
<td>1</td>
<td>DMEM</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>DMEM + PTOX</td>
<td>11.5</td>
</tr>
<tr>
<td>2</td>
<td>DMEM</td>
<td>4.3</td>
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<td></td>
<td>DMEM + PTOX</td>
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<tr>
<td>3</td>
<td>DMEM + PTOX</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>DMEM+PTOX</td>
<td>12.5</td>
</tr>
</tbody>
</table>
Figure 8. Effect of PTOX on PGE_2 stimulated adenylyl cyclase activity in whole cells. The UMR 106–01 cells were grown in 24–well plates and treated as described under "Methods". The experiment was performed in the absence of serum with or without pertussis toxin (50 ng/ml). The differences seen were statistically significant (p< 0.005 at 10^{-6} M PGE_2). The values represent the mean and s.d. of triplicate determinations within this experiment. The protein values were determined to be similar between PTOX treated and untreated cells, using Lowry protein assay.
3.3. Identification of G Proteins Involved in AC Modulation

Having established that AC responsiveness in UMR 106–01 cells can be modulated by PTOX treatment I proceeded to characterize the G proteins present in these cells that could affect AC activity. Using Western Blot analysis (Fig. 9), it was shown that UMR cells contain Gs (52 and 44 KDa), Gi (40 KDa) and Go (39 KDa) type proteins. These proteins were identified using specific antibodies (which have previously characterized) as well as by assessing their migration on the gel compared to molecular weight standards. The proteins identified in these cells are potential regulators of AC activity (see introduction).

It is also important to note that this is the first report indicating the existence of Go type proteins in these cells. There seems to be more Go in these cells than Gi, as seen by both Western blot analysis and ADP-ribosylation gels (data not shown).

3.4. Effect of Acute PTOX Treatment on AC Activity

Using membrane AC assays I attempted to answer the question of whether the increase in AC response seen, in the whole cell system, could be explained by knocking out Gi/Go mediated inhibition of AC. Acute membrane PTOX treatment did not show the same PTOX effect as seen in the whole cell condition (Fig. 10). Membranes acutely treated with PTOX did not show increases in PTH stimulated AC activity. In all cases, PTH stimulation led to approximately a 2 fold adenylyl cyclase response compared to $10^{-8}$ M GTPγS activity (PTH stimulations carried out in the presence of $10^{-8}$ M GTPγS) in each membrane sample (Table 5).
Figure 9. Identification of the potential proteins modulating adenylyl cyclase activity. Membranes were prepared from UMR 106-01 cells, 50 μg membrane samples were run on SDS–Page gels and analyzed by Western blot using antibodies for Gsα, lane 1; Gα₁, lane 2; and Gα, lane 3.
Figure 10. Effect of acute pertussis toxin treatment on UMR 106-01 membranes. Control membranes (CON) and membranes acutely treated with PTOX (AP) were stimulated using $10^{-8}$ M GTP\textsubscript{y}S (A), $10^{-7}$ M PTH $+10^{-8}$ M GTP\textsubscript{y}S (B) and $10^{-6}$ M forskolin (C). The results are reported as fold stimulation compared to basal activity in the absence of stimulators. The basal activity for the control and acutely PTOX treated membranes were 1.3 and 0.8 pmoles of ATP hydrolyzed/mg/min, respectively. A two fold adenylyl cyclase response was seen with PTH compared to $10^{-8}$ GTP\textsubscript{y}S basal.
Table 5. Effect of acute PTOX treatment on UMR 106–01 membranes. The numbers are expressed as pmoles of ATP hydrolyzed/mg/min for basal and the other numbers represent fold stimulation compared to basal (see Fig. 10 for more details).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Stimulators</th>
<th>Expt# 1</th>
<th>Expt# 2</th>
<th>Expt#3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basal</td>
<td>1.3</td>
<td>1.4</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>10⁻⁸ M GTPγS</td>
<td>6.5</td>
<td>5.2</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>10⁻⁷ M PTH</td>
<td>14.7 (2.3)</td>
<td>12.9 (2.5)</td>
<td>26.3 (2.3)</td>
</tr>
<tr>
<td></td>
<td>10⁻⁶ M Forsk</td>
<td>5.7</td>
<td>4.9</td>
<td>9.4</td>
</tr>
<tr>
<td><strong>Acute PTOX</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basal</td>
<td>0.8</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>10⁻⁸ M GTPγS</td>
<td>6.6</td>
<td>3.2</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>10⁻⁷ M PTH</td>
<td>12.6 (1.9)</td>
<td>5.5 (1.7)</td>
<td>8.2 (2.4)</td>
</tr>
<tr>
<td></td>
<td>10⁻⁶ M Forsk</td>
<td>6.4</td>
<td>1.9</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Note: Fold stimulations are calculated from mean of duplicate determinations. The numbers in brackets represent fold PTH stimulation compared to 10⁻⁸ M GTPγS since PTH stimulations were carried out in the presence of 10⁻⁸ M GTPγS.
The results obtained with GTPγS and forskolin stimulation of the membranes were somewhat variable (Table 5). PTOX treated membranes either showed similar or decreased responsiveness to both GTPγS and forskolin, as compared to the control membranes. This may be due the decrease in the amount of βγ subunits available from Gi/Go type proteins which may be involved in conditionally stimulating AC (see discussion).

In order to test whether acute PTOX treatment had knocked out Gi/Go type proteins, the acutely treated membranes were ADP-ribosylated in the presence of [32P] NAD and run on a SDS-PAGE gel (see "Methods"). Fluorimetric analysis of the gel demonstrated that the acutely treated membranes showed decreased ADP-ribosylation compared to control membranes. This indicates that the acutely PTOX treated membranes had been ribosylated under the conditions used for the membrane adenylyl cyclase assay (refer to "Methods").

These results suggest that the PTOX effect seen in the whole cell is mediated by factors other than the knock out of Gi/Go type proteins. Perhaps PTOX induces some long term changes in these cells that render them more responsive to both PTH and PGE₂ stimulation. To test this, studies were done to assess the effects of PTOX on cell growth.

3.5. The Growth Effects of PTOX

Thymidine incorporation is an index of mitogenic potential. [³H] thymidine assays were used to assess the effects of PTOX on growth. The results demonstrate that untreated cells show very little serum stimulation compared to cells treated in the absence of serum (Fig. 11 and Table 6). The serum stimulation seen under control conditions (untreated) was variable. For instance serum was stimulatory in Fig. 14. The reasons for this variability is unclear. On the other hand,
Figure. 11. Effects of PTOX on [³H] thymidine incorporation in whole cells. The cells were treated as described in "Methods". The DPM values presented were normalized using average protein values determined either by amido black or Lowry protein assay. The values represent the mean and s.d. of quadruplicate determinations within this experiment. The thymidine incorporation seen in the absence of serum represents the basal level. The decrease in the basal level of [³H] thymidine incorporation seen in the presence of PTOX was statistically significant compared to the basal level of incorporation seen in the absence of PTOX (p< 0.005).
Table 6. Effects of PTOX on $[^3]$H thymidine incorporation in whole cells. The numbers represent normalized DPM values from quadruplicate determinations in each experiment. (−S = no serum; S = serum [5% FCS]). See Fig. 11 for more details.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Expt# 1</th>
<th>Expt# 2</th>
<th>Expt# 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>−S</td>
<td>10062 ± 517</td>
<td>39602 ± 7968</td>
<td>78013 ± 24081</td>
</tr>
<tr>
<td>+S</td>
<td>13154 ± 737</td>
<td>44804 ± 3047</td>
<td>75281 ± 10737</td>
</tr>
<tr>
<td>−S +PTOX</td>
<td>2776 ± 284</td>
<td>18563 ± 3079</td>
<td>19202 ± 4555</td>
</tr>
<tr>
<td>+S +PTOX</td>
<td>5850 ± 1699</td>
<td>40593 ± 5125</td>
<td>74798 ± 7407</td>
</tr>
</tbody>
</table>
Figure. 12. Effect of PTOX on cellular growth. Cells were treated with or without PTOX in serum free medium, or with medium containing 5% FCS without PTOX for 3 days. Each day cell numbers were assessed by the protein content in each well as outlined in "Methods".
Table. 7. Effect of PTOX on cellular growth. See Fig. 12 for more details.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% FCS</td>
<td>1.32 ± 0.156</td>
<td>2.09 ± 0.0053</td>
<td>4.29 ± 0.430</td>
</tr>
<tr>
<td>No Serum</td>
<td>8.76 ± 0.0084</td>
<td>1.50 ± 0.141</td>
<td>2.49 ± 0.338</td>
</tr>
<tr>
<td>PTOX</td>
<td>0.754 ± 0.0096</td>
<td>1.01 ± 0.104</td>
<td>1.55 ± 0.273</td>
</tr>
</tbody>
</table>
in the PTOX treated cells a better serum stimulation can be seen. This is mainly because PTOX treatment causes a significant decrease in the basal level of thymidine incorporation. Therefore, it appears that PTOX does affect the serum–independent growth of these cells.

To further assess this growth inhibitory effect of PTOX, the growth of the cells was observed for three sequential days in culture, under three different conditions: 1) in the presence of 5% fetal calf serum (FCS); 2) in the absence of serum; and 3) with PTOX in the absence of serum. As can be seen in Fig. 12 and Table 7, the cells in 5% FCS grew the fastest with 6.28 times the number of original cells after 3 days. In the absence of serum, the cells were still able to grow well, increasing to 3.65 times the original number. But when PTOX was also added, the cells only increased to 2.27 times the original number, after 3 days. Again, this shows that PTOX affects the growth of these cells.

3.6. Mechanism of action of PTOX

As outlined in the introduction, osteoblasts secrete growth factors into the culture medium. It is these autologous growth factors that contribute to cell growth seen under serum free conditions. These observations raise the question of whether PTOX affects the growth of these cells by decreasing the production of autologous growth factors (AGFs)? Alternatively, the effect of PTOX may be mediated by the attenuation of cellular response to their AGFs? To investigate these possibilities, experiments were performed to first establish that these cells produce and secrete their own growth factors and then to determine the effect of PTOX on this production. As shown in Fig. 13 and Table 8, when cells were left serum free for 24 hours, thymidine incorporation was no different than that seen in cells with 10% serum, indicating serum–
Figure 13. Production of autologous growth factors by UMR 106-01 cells. The cells were treated as described in "Methods". The medium was changed 24 hours prior to the $[^3]$H thymidine assay. The control (no treatment) cells were put in serum containing medium and the 1 hour and 24 hour cells were put in serum free medium. The 1 hour cells had their medium changed every hour for 12 hours prior to the assay. The difference between the 1 hour and the control cells was statistically significant ($p < 0.0005$). The DPM values reported were normalized for protein (see Figure 11) and represent the mean and s.d. of quadruplicate determinations within this experiment.
Table 8. Production of autologous growth factors by UMR 106–01 cells. The numbers represent normalized DPM values from triplicate determinations. See Figure 13 for more details.

<table>
<thead>
<tr>
<th></th>
<th>1 hour</th>
<th>24 hour</th>
<th>no treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4049 ± 342</td>
<td>7402 ± 877</td>
<td>6192 ± 468</td>
</tr>
</tbody>
</table>
independent or autologous growth. When serum–free medium was replaced every hour for 12 hours prior to the assay thymidine incorporation was, however, significantly lowered. This finding suggests that the cells grown in serum–free medium for 24 hours were able to condition their medium with AGFs and grow at a similar rate as the cells cultured in serum–containing medium.

Experiments were then performed to test whether PTOX inhibits the ability of UMR 106–01 cells to produce their own growth factors. Serum–free conditioned medium was generated from both PTOX treated and untreated cells (24 hours), put on a new batch of cells and their thymidine incorporation measured. Fig. 14 and Table 9 show that CM from untreated (CM1) cells was able to stimulate thymidine incorporation. Conditioned medium from cells treated with PTOX (CM2) was also stimulatory compared to control. CM1 showed higher stimulation compared to CM2 in all experiments, however, this difference was not statistically significant in 2 of the 3 experiments performed and in all experiments CM2 was clearly stimulatory. Therefore, it was concluded that PTOX does not affect the ability of these cells to produce AGFs but does affect their ability to respond to these AGFs.

Various attempts were made to determine which of the AGFs produced by these cells was the target of PTOX inhibition. However, due to the complexity of the system this was not possible. Some of the growth factors tested were epidermal growth factor (EGF), insulin–like growth factor I (IGFI) and insulin–like growth factor II (IGFII). The cells were unresponsive to addition of individual exogenous growth factor stimulation as measured by [³H] thymidine incorporation. This is probably because the autologous production of their own growth factors acted as a confounding variable in these experiments. Alternative experimental protocols need to be devised in order to test the effect of individual growth factors on these cells. The effect of
Figure. 14. Effect of PTOX on the autologous growth factor production of UMR 106-01 cells. The cells were treated as described in "Methods". The conditioned medium from untreated cells (CM1) and PTOX treated cells (CM2) were put on a new batch of cells and their [³H] thymidine incorporation measured. The difference between CM1 and CM2 was not statistically significant, in this experiment (p> 0.05), as determined by the two tailed t-test. The values represent mean and s.d. of quadruplicate determinations in this experiment.
Table 9. Effect of PTOX on the autologous growth factor production of UMR 106-01 cells. Only Expt#3 showed statistically significant difference between CM1 and CM2 (p< 0.05 for two tailed t-test). See Figure 14 for details.

<table>
<thead>
<tr>
<th></th>
<th>no serum</th>
<th>serum</th>
<th>CM1</th>
<th>CM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt# 1</td>
<td>4137 ± 305</td>
<td>6925 ± 564</td>
<td>10588 ± 4827</td>
<td>7054 ± 428</td>
</tr>
<tr>
<td>Expt# 2</td>
<td>23091 ± 3203</td>
<td>37821 ± 2935</td>
<td>119572 ± 7449</td>
<td>101031 ± 11457</td>
</tr>
<tr>
<td>Expt# 3</td>
<td>1667 ± 95</td>
<td>3736 ± 602</td>
<td>3678 ± 612</td>
<td>2191 ± 155</td>
</tr>
</tbody>
</table>
individual growth factors on these cells were not pursued any further. Therefore I proceeded to assess the effect of cellular growth on the ability of these cells to respond to stimulators of AC. The rationale behind this set of experiments was that if PTOX increases AC responsiveness by slowing down the growth of these cells, then decreasing the growth rate of these cells by some other means should mimic this effect.

3.7. Growth effects on AC responsiveness

In order to study the effect of cellular growth on the AC responsiveness, UMR 106–01 cells were grown in two different types of media: 1) low growth medium (10% NCS in DMEM); and 2) high growth medium (5% FCS in 50:50 DMEM:Hams F12). Cells cultured under high growth conditions grew at least 10 times faster than the low growth cells. The results (Fig. 15 and Table 10) show that the cells grown in low growth medium are more responsive to PTH compared to the cells grown in high growth medium. The generality of this growth effect was assessed. As shown in Fig. 15b, PGE₂ stimulated AC activity was increased in cells cultured in low growth medium. Therefore, once again this effect of increasing AC response is not unique to PTH.

Since the cells cultured in high growth medium are present at higher cell density when assayed than low growth cells, this raises the question of whether the effects seen were due to the differences in density of these cells. To test this possibility, AC assays were performed on cells plated at different cell densities which ranged from 6.25x10³ to 5x10⁴ cells/well. These experiments revealed that as cell numbers increased the amount of cAMP produced per well was proportionally increased. Therefore, a 2-fold stimulation by PTH (10⁻⁸ M), relative to basal
Figure. 15. Changes in whole cell adenylyl cyclase activity under different growth conditions. Adenylyl cyclase response was measured as outlined in "Methods". a) PTH dose–response curves under high and low growth conditions; and b) comparison of $10^{-7}$ M PTH and $10^{-6}$ M PGE$_2$ mediated stimulation of adenylyl cyclase activity, under high and low growth conditions. The values represent the mean and s.d. of triplicate determinations within this experiment and they have been corrected for protein.
Table 10. Changes in whole cell adenylyl cyclase activity under different growth conditions. 5% FCS= high growth; 10% NCS=low growth. See Fig. 15 for more details.

<table>
<thead>
<tr>
<th>Expt#</th>
<th>Treatment Condition</th>
<th>Fold Stimulation (10 nM PTH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5% FCS</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>10% NCS</td>
<td>9.2</td>
</tr>
<tr>
<td>2</td>
<td>5% FCS</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>10% NCS</td>
<td>11.0</td>
</tr>
<tr>
<td>3</td>
<td>5% FCS</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>10% NCS</td>
<td>7.4</td>
</tr>
</tbody>
</table>
activity, was seen at all the different densities assayed (data not shown). Thus the results support the hypothesis that slowing down the growth of the UMR 106–01 cells leads to increased AC responsiveness, independent of cell density. The mechanism of this effect was further investigated using various agents to stimulate AC at post- receptor loci.

3.8. Mechanism of Growth Effects

To further explore the mechanism(s) underlying the differences in AC responsiveness induced by growing the cells in different media, membrane adenylyl cyclase assays were performed (Fig. 16 and Table 11). These assays show that both GTPyS and forskolin stimulation of AC activity were comparable in membranes prepared from cells grown in both high and low growth medium. These results suggest that the changes that are induced by growing the cells in different media are not occurring at the level of G proteins or adenylyl cyclase.

Further assessment of G protein levels was performed using Western blot analysis on both high and low growth cell membranes (Fig. 17). These results demonstrated that the levels of Gs were clearly not increased under the two conditions.

The conclusion drawn from both the membrane adenylyl cyclase assays and Western blot analysis was that post receptor components of AC stimulation are not changed, under low growth conditions.

3.9. Results Summary

The results of my experiments demonstrate that adenylyl cyclase responsiveness of UMR 106–01 osteoblasts can be profoundly enhanced by treating whole cells with PTOX. This effect
Figure 16. Membrane adenylyl cyclase assay of cells grown in high and low growth medium. Membranes were prepared from cells grown in high and low growth medium and adenylyl cyclase activity was measured as outlined in the "Methods". The membranes were stimulated with $10^{-7}$ M GTPyS (A) and $10^{-6}$ M forskolin (B). The results are reported as fold stimulation compared to basal activity in the absence of stimulators. The basal activity for the high and low growth cell membranes were 2.8 and 1.1 pmoles of ATP hydrolyzed/mg/min (mean of duplicate determinations), respectively.
Table 11. Membrane adenylyl cyclase assay of cells grown in high and low growth medium. The numbers are expressed as pmol of ATP hydrolyzed/mg/min. They represent the mean of duplicate determinations. See Fig. 16 for more details.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Stimulators</th>
<th>Expt# 1</th>
<th>Expt# 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NCS</td>
<td>Basal</td>
<td>1.1</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>$10^{-8}$ M GTPγS</td>
<td>16.7</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>$10^{-7}$ M GTPγS</td>
<td>18.6</td>
<td>19.3</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}$ M Forsk</td>
<td>12.5</td>
<td>13.9</td>
</tr>
<tr>
<td>5% FCS</td>
<td>Basal</td>
<td>2.8</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>$10^{-8}$ M GTPγS</td>
<td>34.1</td>
<td>31.9</td>
</tr>
<tr>
<td></td>
<td>$10^{-7}$ M GTPγS</td>
<td>44.7</td>
<td>33.8</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}$ M Forsk</td>
<td>31.1</td>
<td>22.2</td>
</tr>
</tbody>
</table>
Figure 17. Effect of high and low growth medium on Gsα protein levels in UMR cell membranes. Membranes were prepared from cells grown in low growth (lanes 1–4) and high growth (lanes 5–8) medium. 10, 20, 40 and 60 μg samples were run on an 11% polyacrylamide gel, transferred to nitrocellulose and probed for Gsα protein as described in "Methods".
is not due to knock out of Gi–inhibition of adenylyl cyclase but appears to be an indirect consequence of PTOX inhibition of cell growth. My experiments can be explained by assuming that this is mediated by PTOX–inhibition of cell proliferation induced by autologous growth factors. The effect of PTOX is mimicked by growth medium that slows down UMR cell growth. The exact mechanism of action of PTOX and the low growth medium in enhancing both PTH and PGE₂ mediated stimulation of AC still remains to be determined, however, it is clear that these growth inhibitory agents are not acting at the post–receptor level.
4. DISCUSSION

The work outlined in this thesis attempted to determine the mechanisms by which the adenylyl cyclase system of osteoblastic cells can be regulated. In particular, experiments were directed towards determining the mechanism(s) by which pertussis toxin increases the adenylyl cyclase response to PTH in these cells.

The data presented in my initial experiments demonstrated that inhibition of Gi/o proteins in UMR106-01 cells by PTOX treatment of the whole cells, led to substantial increases in the ability of these cells to respond to PTH. This result is similar to previous reports of the effects of PTOX on ROS17/2.8 cells by Pines et al. (1986) and Abou-Samra et al. (1989b), and indicates that Gi/o proteins play a role in modulating AC responsiveness in osteoblastic cells. There are several potential mechanisms by which inhibition of Gi/o proteins could increase stimulation of AC mediated by receptor activation of Gs.

The first, proposed by Pines et al. (1986), is that Gi proteins are tonically active in these cells and thereby attenuate stimulatory signals from Gs. If this were the case, PTOX inhibition of Gi proteins should increase basal cAMP levels in the cells as well as those seen following receptor activation. Data from my experiments did not support this mechanism since in the majority of experiments no increase in basal AC activity was seen under conditions that increased PTH and PGE\textsubscript{2} responses.

The second possibility, suggested by Pines et al to explain the effect of PTOX on PTH-stimulated adenylyl cyclase in the absence of any effect on basal activity, proposed that the PTH receptor is coupled to both Gs and Gi. Although this proposal does offer an explanation for the effects of PTOX on the cells, several of my experiments refute this possibility. In particular,
when Gi/o proteins were acutely inhibited in membranes there was very little effect on basal or PTH– activated AC, suggesting that AC in these cells is not inhibited by Gi/o. Indeed this would be consistent with a recent report in which it was noted that osteoblasts express type IV AC (Ish–Shalom et al., 1996) an AC subtype which is not inhibited by Gi proteins.

Since neither of these previously proposed mechanisms were supported by my data, and the PTOX effect was found not to be specific to PTH but also increased PGE₂–stimulated AC, it suggested that PTOX has a more general effect on these cells. Therefore, the possibility that PTOX treatment may have other effects on the cells that could lead indirectly to regulation of AC was investigated.

It was noted that one of the effects of PTOX is profound inhibition of the growth of UMR106–01 cells. This effect was explored further and the results suggest that PTOX inhibits serum–independent growth of the cells mediated by their own secreted growth factors. Osteoblasts produce a number of growth factors (as outlined in the introduction) and my experiments suggested that PTOX does not inhibit the production of these growth factors but rather alter the ability of the cells to respond to them. The mechanism of this inhibition is not obvious, since most of the growth factors secreted by osteoblasts act via receptors of the tyrosine kinase family that do not couple to G proteins. However, UMR106–01 cells have been shown to produce large amounts of IGFII (Mohan et al., 1990) and express IGFII receptors has been shown to couple to Gi₂ (Lange–Carter et al., 1994, Okamoto et al., 1990). As shown in Fig. 18, the IGFII receptor is a monomeric transmembrane protein, which couples via Gi₂ to the Raf1 protein and feeds into the mitogen activated protein kinase (MAPK) pathway (Lange–Carter et al., 1994). Therefore, it is plausible that osteoblastic IGFII activates the MAP kinase pathway via
Figure 18. Schematic representation of the IGFI and IGFII receptors. Both of these receptors feed into the MAPK pathway via the raf 1 protein (modified from LeRoith et al., 1994).
IGFIIR/Gi₂ and PTOX inhibits this autocrine loop by knocking out Gi₂ in these cells. Numerous attempts were made to test this hypothesis more directly by determining the effect of PTOX on exogenous IGFII-stimulated growth. However, the autologous production of growth factors by the cells confounded these experiments, and the precise mechanism of PTOX inhibition of osteoblastic growth remains to be determined. Nevertheless, my experiments have clearly demonstrated that PTOX alters osteoblastic growth possibly as a result of decreased responsiveness to their own growth factors. It remained to be determined if growth inhibition is responsible for the increased responses to PTH and PGE₂ seen in the adenylyl cyclase assays.

To investigate the effect of modulating cellular growth on adenylyl cyclase responsiveness, a number of different media and serum supplements were tested on the UMR106–01 cells. Two combinations of tissue culture medium and sera were found, one that supported rapid growth of the cells and another which maintained the cells at a lower growth rate. Results of a series of adenylyl cyclase assays demonstrated that cells grown in the low growth media had profoundly increased responses to PTH and PGE₂, when compared to those grown in high growth media. Indeed, recent experiments have shown that over the course of four subcultures into high growth medium, UMR106–01 cells loose their ability to respond to PTH or PGE₂ almost completely (Mayeenuddin and Pang, unpublished data). This effect does not appear to be the result of dedifferentiation of the cells since their ability to respond robustly to PTH can be restored by acutely placing the cells into low growth media. Therefore it was hypothesized that some component of the adenylyl cyclase pathway was inhibited by the high growth medium. Since the effect was common to both PTH and PGE₂ responsiveness, I examined the common post-receptor components of the adenylyl cyclase system in the cells. Using
membrane adenylyl cyclase assays the activation of Gs and the catalytic unit of adenylyl cyclase were directly probed with guanine nucleotide analogs and forskolin, respectively. Neither of these responses were altered by growing the cells in high and low growth media. Furthermore, Gsα protein levels were not altered by these two growth conditions as assessed by immunoblotting techniques. The conclusion that must be drawn from these experiments is that decreases in PTH and PGE₂ activation of adenylyl cyclase in high growth medium are the result of changes at the level of the receptors. The mechanism of receptor desensitization in high growth medium has not been explored here and should be the subject of future studies. I suggest that two possible mechanisms may account for this observation and both of these could be tested in the future studies.

First, the expression of PTH and PGE₂ receptors may be altered in the cells in response to changes in the medium. This could be tested by assessing the mRNA levels for the PTH and PGE₂ receptors as well as direct binding assays using radiolabelled ligands. Second, the receptors may not be decreased in number but may be desensitized by receptor phosphorylation. A mechanism for this phosphorylation is suggested by recent studies by Karoor and Malbon (1996), in which they demonstrated IGFI-receptor mediated phosphorylation of the β₂-adrenergic receptor. Their experiments showed that IGFI treatment abrogates isoproterenol-mediated activation of cAMP accumulation in the cells. Therefore it is plausible that the PTH and PGE₂ receptors in UMR106-01 cells are also phosphorylated by kinases activated by growth factors in the medium. Thus in the high growth medium in which the growth factors supplied by fetal serum supplements is increased, the receptors would be phosphorylated and therefore desensitized. It is also possible that the growth factors secreted by the cells also act in this way
to inhibit responses through PTH and PGE\textsubscript{2} receptors. Phosphorylation of the receptors should be assessed in these cells in future studies.

Although the mechanism of action of the medium effect is still not known, my studies show that this is a good system to study the growth environment of osteoblasts. The results show that a clonal cell line can demonstrate a wide range of AC responsiveness depending on the type of growth environment it is exposed to. This finding may be important in trying to understand the effects of PTH \textit{in vivo}, both in normal and pathophysiological conditions. It is well known that bone cells secrete growth factors that are stored in the bone matrix. These growth factors are thought to be important in coupling bone resorption to bone deposition. In light of the data presented here, changes in the growth factor composition in the microenvironment of the bone cells may also lead to vast differences in PTH responsiveness. For instance, if the amount of growth factors present were increased this may lead to a decrease in PTH responsiveness and therefore a possible decrease in bone resorption in response to PTH. On the other hand, if the amount of growth factors present were decreased this might lead to increased responsiveness to PTH and thus lead to greater bone resorptive activity. In general, PTH responsiveness seems to be inversely related to growth factor content.

Interestingly, in patients suffering from osteoporosis, the circulating levels of IGFs and IGF binding proteins have been shown to be decreased. IGFI is known to mediate the actions of growth hormone in bone (Wuster et al., 1993; Schmid et al., 1989; Stracke et al., 1984) and IGF binding protein 3 (IGFBP–3) acts to potentiate the biological actions of IGFI. IGFI (Mohan et al., 1991b; Ljunghall et al., 1992), IGFI (Nakamura et al., 1990) and IGFBP–3 levels have been shown to be decreased in patients with osteoporosis (Wuster et al., 1993) and my experiments
suggest that under these conditions osteoporotic patients may be more susceptible to PTH stimulated bone resorption. A reduction in the circulating levels of IGFs, however, does not necessarily reflect what is going on at the level of local bone growth factors. This area requires more research. The UMR 106-01 high and low growth cell model established in this study may be helpful in understanding how changes in the composition of local bone growth factors can modulate osteoblast responsiveness to bone resorptive agents, such as PTH.

The data presented here, clearly shows that long term PTOX treatment on whole cells not only leads to ADP-ribosylation of Gi/Go type proteins but also to changes in cellular growth. In light of this information, earlier studies of PTOX mediated increases in adenylyl cyclase responsiveness, which utilized the method of long term PTOX treatment, should be interpreted with caution.

In addition, although the majority of my discussion has focused on the potential involvement of Gi proteins in modulating osteoblastic cell proliferation, it should be noted that UMR 106-01 cells also express large amounts of Go proteins. In fact as mentioned earlier, they express more Go than Gi type proteins. The role of Go proteins in UMR 106-01 cellular growth regulation has yet to be determined. Go is most abundant in the brain where it is believed to be involved in the inhibition of voltage-activated calcium channels (Rouot et al., 1987), and Go proteins may interact with calcium channels present in bone cells. Recent studies, however, have also shown Goα mediated stimulation of the MAPK pathway via a protein kinase C-dependent mechanism (Biesen et al., 1996). Therefore, it is possible that Go proteins in UMR 106-01 cells play a role in the activation of the MAPK pathway and therefore cellular growth regulation. Again, more studies are required to determine the role of Go proteins in bone cells.
5. FUTURE STUDIES

To better understand adenylyl cyclase modulation in these cells, future work should be directed towards identifying the regulatory components of the AC system that are present in these cells. The type of Gi proteins expressed in these cells should be assessed, as well as the subtype(s) of adenylyl cyclase. Effort should also be made in studying receptor expression under different growth conditions. Both binding studies and the receptor mRNA levels should be tested.

In addition, the importance of IGFII and the other autologous factors released by the UMR 106-01 cells should be determined. Either anti-IGFII antibodies and/or anti-IGFII receptor antibodies may be used to show if IGFII is essential for optimal UMR 106-01 cell proliferation. Antisense studies could be done to determine if specific Gi2 and IGFIIR knock outs can mimic the PTOX effect seen in these cells.
6. REFERENCES


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