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Modulation of parathyroid hormone 1 receptor signaling by extracellular nucleotides

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ABSTRACT

Parathyroid hormone (PTH) activates the PTH/PTH-related peptide receptor (PTH1R) on osteoblasts and other target cells. Mechanical stimulation of cells, including osteoblasts, causes release of nucleotides such as ATP into the extracellular fluid. In addition to its role as an energy source, ATP serves as an agonist at P2 receptors and an allosteric regulator of many proteins. We investigated the effects of concentrations of extracellular ATP, comparable to those that activate low affinity P2X7 receptors, on PTH1R signaling. Cyclic AMP levels were monitored in real-time using a bioluminescence reporter and β-arrestin recruitment to PTH1R was followed using a complementation-based luminescence assay. ATP markedly enhanced cyclic AMP and β-arrestin signaling as well as downstream activation of CREB. CMP – a nucleotide that lacks a high energy bond and does not activate P2 receptors – mimicked this effect of ATP. Moreover, potentiation was not inhibited by P2 receptor antagonists, including a specific blocker of P2X7. Thus, nucleotide-induced potentiation of signaling pathways was independent of P2 receptor signaling. ATP and CMP reduced the concentration of PTH (1-34) required to produce a half-maximal cyclic AMP or β-arrestin response, with no evident change in maximal receptor activity. Increased potency was similarly apparent with PTH1R agonists PTH (1-14) and PTH-related peptide (1-34). These observations suggest that extracellular nucleotides increase agonist affinity, efficacy or both, and are consistent with modulation of signaling at the level of the receptor or a closely associated protein. Taken together, our findings establish that ATP enhances PTH1R signaling through a heretofore unrecognized allosteric mechanism.

Key words – adenosine 5’-triphosphate (ATP), cyclic adenosine 3’,5’-monophosphate (cAMP), cyclic adenosine 3’,5’-monophosphate response element binding protein (CREB), cytidine 5’-monophosphate (CMP), extracellular nucleotides, G protein-coupled receptor (GPCR), parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R), positive allosteric modulator (PAM), potentiation

CO-AUTHORSHIP

Chapter 1 entitled “INTRODUCTION” was written by B. H. Kim with suggestions from Drs. P. Chidiac and S. J. Dixon.

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Chapter 3 entitled “GENERAL DISCUSSION AND CONCLUSIONS” was written by B.H. Kim with suggestions from Drs. P. Chidiac, S. J. Dixon.

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Since November of 2013, it has been 4 years and 7 months. During this long period, I transitioned back and forth between volunteer, workstudy student, summer student, and thesis student until eventually becoming a graduate student.

Many good things happened: We made discoveries, we made friends to hang out for good lunches and dinners. We helped (and were helped) many in pursuit of their goal. I have upgraded my computer greatly.

There were equally many bad things: My father passed away. Experiments just simply didn't work. Several machines and equipment, without warning, failed. Worst of all, (I now confess) beakers were shattered due to my carelessness.

Importantly though, I made it through and learned much in the process, and that would not have been possible without the support from many. To acknowledge a few: My father, who now rests in heaven, for his sacrificial commitment for the good of the family; mother and my brother Timothy Adrian Thonkmo, for their tireless works for the same reason. Friends Eric, Will, Thai-son, and Brian for their morale support. Colleagues Ryan Beach, Alexey Pereverzev, Yara Hosein, Tom Chrones, and Anette Surmanski for all the beers and quotes. Supervisors Drs. Stephen Sims, S. Jeffrey Dixon, and Peter Chidiac for opportunities and teachings. Advisory members Drs. Peter Stathopulos, Cheryl Seguin, and Nica Borradaile for advises. Thank you.

"*It's not what you say, it's how you say it*" – Ryan Beach

"*You've got to risk it for the biscuit*" – Someone

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$[Ca^{2+}]_i$	cytosolic free Ca^{2+} concentration
A438079	3-(5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl)methyl pyridine hydrochloride
AC	adenylyl cyclase
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
AMP-PNP	adenosine 5'-(β,γ-imido)triphosphate
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
BMD	bone mineral density
BMU	basic multicellular unit
BSA	bovine albumin
BSP	bone sialoprotein
CaMKII	calmodulin-dependent protein kinase II
cAMP	adenosine 3',5'-cyclic monophosphate
CIHR	Canadian Institutes of Health Research
CMP	cytidine 5'-monophosphate
COL1	type 1 collagen
CRE	cAMP response element
CREB	cAMP response element-binding protein
c-Src	cellular proto-oncogene tyrosine-protein kinase src
CTP	cytidine 5'-triphosphate
ERK	extracellular signal-regulated kinase
F16BP	fructose 1,6-bisphosphate

F6P	fructose 6-phosphate
FDA	United States Food and Drug Administration
G1P	glucose 1-phosphate
G6P	glucose 6-phosphate
Ga1P	galactose 1-phosphate
GABA	γ-aminobutyric acid
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
Gi	inhibitory G protein
GIRK	G protein-coupled inwardly-rectifying K^+ channel
GM-CSF	granulocyte/macrophage-colony stimulating factor
GPCR	G protein-coupled receptor
Gq	G protein q
Gs	stimulatory G protein
GTP	guanosine 5'-triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α	hypoxia-induced factor 1α
IBMX	3-isobutyl-1 methylxanthine
indo-1-AM	indo-1-acetoxymethyl ester
IP_3	inositol 1,4,5-trisphosphate
LRP	lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MEK	MAPK/ERK kinase

MMP9	matrix metallopeptidase 9
NAM	negative allosteric modulator
NF-κB	nuclear factor kappa B
OCN	osteocalcin
ON	osteonectin
OPG	osteoprotegerin
OPN	osteopontin
OSX	osterix
P2X	ATP-gated P2X receptor cation channel family
P2Y	purinergic G protein-coupled receptors
PAM	positive allosteric modulator
PBS	phosphate-buffered saline
P_i	inorganic phosphate
PI3K	phosphatidylinositol-3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PLC	phospholipase C
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PTH	parathyroid hormone
PTH (1-14)	amino-terminal fragment of parathyroid hormone (1-14)
PTH (1-34)	amino-terminal fragment of parathyroid hormone (1-34)
PTH1R	parathyroid hormone/parathyroid hormone-related peptide receptor
PTHrP	parathyroid hormone-related peptide
Raf	proto-oncogene serine/threonine-protein kinase Raf

RANK	receptor activator of nuclear factor kappa B
RANKL	receptor activator of nuclear factor kappa B ligand
R5P	ribose 5-phosphate
Ro 20-1724	4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one
RUNX2	runt-related transcription factor 2
SEM	standard error of the mean
TRAP	tartrate-resistant acid phosphatase
TTP	thymidine 5'-triphosphate
UDP	uridine 5'-diphosphate
UDPG	uridine 5'-diphosphate glucose
UTP	uridine 5'-triphosphate
V-ATPases	vacuolar-type H^+ ATPase
VEGF	vascular endothelial growth factor
Wnt	wingless/integrated

CHAPTER ONE

INTRODUCTION

1.1 Overview of chapter one

This chapter is divided into four sections. In the first section, bone physiology will be discussed with foci on bone cells, skeletal development and bone remodeling. Also, pathological conditions associated with poorly regulated bone remodeling will be briefly discussed. The second section will discuss about G protein-coupled receptor (GPCR). This section will have emphases on signaling, regulation and allosteric modulation of GPCR. In addition, parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) will be discussed with foci on physiological function, associated pathologies, and pharmacological intervention of osteoporosis. The third section will discuss about nucleotides. Specifically, the physiology of extracellular nucleotides will be discussed. In the last section, brief research rationale will be provided with objectives.

1.2 Bone physiology

1.2.1 Bone

Bone in vertebrates provide the structural support to soft connective tissues and protection to vital soft organs. As well, bone provides framework for skeletal muscle attachment for motility, while acting as a storage for calcium and phosphate ions to allow for homeostasis (Marks Jr and Odgren, 2002). Human skeletal system consists of over 200 bones, which are, based on shape and function, categorized into one of five types: flat, long, irregular, short, and sesamoid bone. Each bone is an organ that consists of various tissues including a periosteum, endosteum, vasculature, nerve and bone tissue. A mineralized bone tissue is composed of an inorganic and organic phase. Large portion of the bone is composed of the inorganic phase, which is mainly consisted of mainly of calcium and phosphate ions organized in the form of hydroxyapatite crystal ($Ca_{10}(PO_4)_6OH_2$) (Hadjidakis and Androulakis, 2006). The rest of bone is composed of an organic phase, which consists primarily of type I collagen (COL1). Only a smaller fraction of organic phase (~10%) is composed of non-collagenous proteins, such as osteocalcin (OCN), osteonectin (ON), osteopontin (OPN) and bone sialoprotein (BSP) (Duong et al., 2002; Robey et al., 1993).

Bone has external compact bone and internal trabecular bone. Compact bone provides the structural integrity of the bone and accounts for 80% of bone tissue weight (Hadjidakis and Androulakis, 2006). Compact bone is composed of basic units called osteon, which is a rod-shaped structure densely packed within the bone. An individual osteon consists of: 1) Haversian canal, an opening at the center with vasculature and nerve; 2) lamellae, a bone tissue that concentrically surround the Haversian canal; and 3) Volkmann canal, which connects

neighbouring Haversian canals (Parfitt, 1994). Trabecular bone is found interior to compact bone and consists of thin mineralized tissue in irregular meshwork pattern, shape of which resembles sponge – thus, trabecular bone is also referred to as a spongy bone. Owing to its shape, trabecular bone has greater surface area to allow for dynamic turnover of bone for calcium phosphate homeostasis (Clarke, 2008).

1.2.2 Bone cells

Removal and formation of bone is achieved by osteoclasts and osteoblasts, respectively. Osteoclasts are large, multinucleated cells that are derived from fusion of haematopoietic osteoclast precursor cells that is promoted by two factors: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL). M-CSF is a proliferative factor that plays a role in earlier phase of the osteoclast development, rather than later differentiation (Teitelbaum, 2000). Mice with inactivating mutant M-CSF develop early onset osteopetrosis; however, these mice eventually recover as granulocyte/macrophage-colony stimulating factor (GM-CSF) compensates for the loss of M-CSF later in development (Niida et al., 1999). Vascular endothelial growth factor (VEGF) was also demonstrated to be able to substitute M-CSF to recruit osteoclasts to sites of resorption, thus indicating that M-CSF is not critical to commitment to osteoclast lineage (Niida et al., 1999; Teitelbaum, 2000). In contrast, RANKL, which is released by osteoblasts as a soluble factor or presented in membrane-bound form, is crucial for osteoclast differentiation (Hsu et al., 1999; Kobayashi et al., 2009; Yasuda et al., 1998). Characterization of skeletal phenotypes in mice with loss of functional RANKL or its receptor RANK revealed lack of osteoclast differentiation and subsequent bone resorption,

causing severe osteopetrosis (Kong et al., 1999). At a cellular level, RANK signaling in osteoclast precursors results activation of c-Src, which in turn activate MAPK and phosphatidylinositol-3-kinase (PI3K). Ultimately, ERK and NF-κB are activated to commit precursors into osteoclast lineage (Wong et al., 1999; Wong et al., 1998). In mature osteoclasts, RANK signaling inhibits apoptosis and promotes survival (Hsu et al., 1999). RANK signaling is suppressed by osteoprotegerin (OPG), a soluble factor released by osteoblasts and osteocytes, that binds to RANKL to inhibit RANKL-RANK interaction (Simonet et al., 1997; Udagawa et al., 2000; You et al., 2008). Consistent with the anti-osteoclastic role of OPG, an *in vitro* study that monitored osteoclast survival revealed that the inhibition of RANK signaling by OPG results in osteoclast apoptosis (Lacey et al., 2000). Furthermore, an *in vivo* study characterizing the skeletal phenotype of homozygous knockout of OPG in mice revealed that the lack of OPG results in development of early-onset osteoporosis and reduced mineralization in alveolar bone (Bucay et al., 1998; Sheng et al., 2010). Not surprisingly, physiological regulation of osteoclast differentiation involves reciprocal modulation of RANKL and OPG expression; for example, parathyroid hormone, which stimulates osteoclast differentiation, increases RANKL while decreasing OPG expression in osteoblasts (Huang et al., 2004).

Bone resorption by functional osteoclast is remarked by formation of sealing zone and ruffled border. Sealing zone is an annular actin network coupled to focal adhesion complex at the basolateral side of osteoclast, and isolates the microenvironment undergoing resorption from extracellular environment (Marchisio et al., 1984; Stenbeck and Horton, 2000). Ruffled border refers to a plasma membrane that is roughly creased within the sealing zone; this irregular shape of the membrane is thought to increase the cellular surface area within the microenvironment (Stenbeck, 2002). During the resorption, osteoclasts first demineralize the inorganic phase of the

bone in the microenvironment by acidifying the zone. Acidification, which is mediated by vacuolar-type H^+ ATPase (V-ATPases) and carbonic anhydrase II, reduce the pH of the microenvironment to be as low as 4.7 (Blair et al., 1989; Silver et al., 1988; Teitelbaum, 2000). To sustain homeostatic bicarbonate concentration and electroneutrality within the cell and the microenvironment during resorption, HCO_3^-/Cl^- exchanger and Cl^- channel are recruited to apical side and ruffled border, respectively (Rousselle and Heymann, 2002). Following the demineralization of the bone surface in microenvironment, cathepsin K, matrix metalloproteinase 9 (MMP9) and tartrate-resistant acid phosphatase (TRAP) are released to degrade the organic phase of bone, which is mainly comprised of collagen (Gowen et al., 1999). Catalytic activity of released cathepsin K and TRAP are optimal in acidic environment, which is achieved by preceded acidification (Blair et al., 1986; Henneberry et al., 1979). Whether the catalytic activity of MMP9 is enhanced by decreasing pH is unknown; however, a cathepsin K-mediated cleavage of MMP9 has been demonstrated to enhance the catalytic activity of MMP9, implying that acidification of the microenvironment also enhances MMP9 activity (Christensen and Shastri, 2015). Osteoclasts eventually stop resorbing in response to signal not yet known, and detach from the matrix to migrate and resorb another region of bone. This resorption cycle of osteoclast continues until apoptosis is triggered (Kanehisa and Heersche, 1988; Vaananen and Horton, 1995; Vaananen and Zhao, 2002).

Mesenchymal stem cells (MSC) are responsible for development of many cell types found in various connective tissues, including bone forming osteoblasts. Commitment of MSC into osteoblast is a process that requires transcription factors runt-related transcription factor 2 (RUNX2) and osterix (OSX). RUNX2 function is required for early commitment into osteogenic lineage. When expressed and activated, RUNX2 upregulate genes related to osteoblast function,

such as OCN, COL1, BSP and OSP. Mice lacking functional RUNX2 develop cartilaginous skeleton absent in osteogenic elements, highlighting the importance of RUNX2 in osteoblast differentiation (Ducy et al., 1997; Komori et al., 1997). OSX expression, which is latent to RUNX2 in differentiation pathway, is another crucial step for osteoblast differentiation. Like RUNX2 mutant mice, mice without functional OSX fail to calcify bones formed via intramembranous processes. Although endochondral skeletons calcify, characterization of skeletal elements in these bones revealed that these bones are devoid of essential components such as OCN, BSP and COL1, suggesting that observed calcification is mediated by hypertrophic chondrocytes rather than osteoblasts (Nakashima et al., 2002).

Activity of matured osteoblasts is regulated by signaling to various receptors, such as Frizzled, a Wnt receptor, and parathyroid hormone/parathyroid hormone-related peptide receptor. In response to activation of these receptors, osteoblasts express components found in bone such as COL1, OCN and ALP, (PTH1R) (Collin et al., 1992; Fermor and Skerry, 1995; Hoang et al., 2004). Ultimately, the role of osteoblasts is to deposit osteoid, a COL1-rich unmineralized organic component of bone that eventually calcifies to form a mineralized connective tissue (Hall and Miyake, 2000). During deposition, osteoblasts are buried under the osteoid during deposition; some of these cells terminally differentiate into osteocytes, which are mechanosensitive cells within bone. Osteoblasts that deposit osteoid at the surface of the bone differentiate into bone lining cells. Osteoblast that do not differentiate undergoes senescence or apoptosis (Manolagas, 2000; You et al., 2008). Osteocytes reside within the space in bone matrix called lacunae and communicate with neighbouring cells via cell processes, which form gap junctions with the cell processes of other cells. These cell processes are found within hollow canal called canaliculus, which are filled by interstitial fluid. Mechanical stimulation of bone is

thought to induce flow of these fluids that is detected by osteocytes and induces electrochemical signaling between osteocytes or with other cell types (Burger et al., 2003; Srinivasan and Gross, 2000).

1.2.3 Skeletal development

The skeletal development in vertebrates is achieved through intramembranous or endochondral ossification. After gastrulation, neural crest and paraxial mesoderm (somites) grow into calvariae of neurocranium, membranous viscerocranium and part of clavicle through intramembranous ossification (Santagati and Rijli, 2003; Tubbs et al., 2012). This process is initiated by clustering of preosteogenic mesenchymal cells to form a cell aggregate known as a condensation, which acts as an early initiation site for bone development (Hall and Miyake, 2000). Once mesenchymal condensation is formed, its further growth is mediated by proliferation of cells within the condensation and new cells are not recruited from the surrounding environment (Yoshida et al., 2008). Mesenchymal cells proliferate and expand until the condensation reaches a predefined limit (Percival and Richtsmeier, 2013). Cells at periphery of condensation continue to proliferate, while centrally located cells undergo osteogenic differentiation. These processes lead to growth of the condensation into a bone spicule, which fuses with other spicules to form a complete trabecula (Percival and Richtsmeier, 2013). Trabeculae interconnect to construct a bone, which takes the primitive shape of the developing skeleton (Collin-Osdoby, 1994). At this stage of bone development, the bone is composed of disorganized matrices, and is known as a woven bone. The Woven bone has poor biomechanical properties and is susceptible to load-induced fractures. Further into development, the woven

bone is remodelled into a lamellar bone. Unlike woven bone, lamellar bone consists of organized collagen matrices in lamellar pattern, which has superior biomechanical properties (Clarke, 2008). Concomitantly, fibrous periosteum and endosteum, which serve as sources for osteoblasts in later development and during bone homeostasis, develop juxtaposing the bone (Karaplis, 2002).

Development of other axial bones (*i.e.* chondrocranium of neurocranium, cartilaginous viscerocranium, ribs, and vertebrae) and appendicular skeleton is mediated by endochondral ossification. Axial bones develop from cranial neural crest and paraxial mesoderm (somites) (Chevallier, 1975; Christ and Wilting, 1992), while appendicular bones develop from lateral plate mesoderm (Winslow et al., 2007). Like intramembranous ossification, endochondral ossification is initiated by mesenchymal condensation, which expands until it reaches the predefined limit. Distinctively, mesenchymal cells differentiate into chondrocytes during endochondral ossification. Cells surrounding the condensation at the periphery differentiate into perichondrial cells. Differentiated chondrocytes proliferate and deposit matrix rich in type II collagen (COL2) and proteoglycan, and a hyaline cartilage takes its initial morphology. The shape of a hyaline cartilage of long bones is similar to that of a staff, with shaft region and rounded ends, which are referred to as the diaphysis and epiphyses, respectively. A small region between the diaphysis and the epiphysis is referred to as a metaphysis. Metaphysis is an area adjacent to the epiphyseal plate, which appear in later during development, located on an end closer to the diaphysis (Karaplis, 2002; Kronenberg, 2003). At certain point of cartilage development, chondrocytes near the center of the diaphysis stop proliferating and undergo hypertrophy in response to signal that remains unknown. Hypertrophic chondrocytes deposit type X collagen instead of type II collagen and mineralize surrounding matrix, but with poorer

biomechanical properties compared to mature bone (Karaplis, 2002; Sun and Beier, 2014). Histomorphometrically, chondrocyte hypertrophy first appears near the center of the diaphysis and spread out toward epiphyses. Chondrocyte proliferation, matrix deposition and hypertrophy contribute to longitudinal lengthening of hyaline cartilage (Hunziker, 1994). As the cartilage grows, chondrocytes in the center are buried and experience hypoxia. These buried chondrocytes express hypoxia-induced factor 1α (HIF-1α), which promotes chondrocyte survival (Schipani et al., 2001). In addition, HIF-1α induces expression of VEGF, which also promote chondrocyte survival while stimulating angiogenesis to the diaphysis. (Filipowska et al., 2017; Maes et al., 2004; Schipani et al., 2001). VEGF also recruit chondroclasts to resorb calcified cartilage synthesized by hypertrophic chondrocytes (Gerber et al., 1999). Osteoblasts are then derived from: 1) perichondrial cells (Lefebvre and Bhattaram, 2010; Nakashima et al., 2002); 2) precursors delivered through the vasculature (Gerber and Ferrara, 2000; Kronenberg, 2006); and 3) hypertrophic chondrocytes that undergo osteogenic differentiation (Yang et al., 2014). Like chondrocytes, osteoblasts that experience hypoxia express HIF-1α to promote osteoblast survival and VEGF expression, which further induces angiogenesis (Steinbrech et al., 1999; Xu et al., 2015). During the formation of primary ossification center, osteoclasts are recruited to resorb woven bone deposited by osteoblasts (Takahashi et al., 1986; Wang et al., 2004). Thus, the bone formation and resorption appear near the center of the diaphysis and spread out toward epiphyses (Kronenberg, 2003). Bone modeling and bone remodeling by osteoclasts and osteoblasts ultimately lead to formation of lamellar compact and trabecular bone and the medullary cavity (Marks Jr and Odgren, 2002).

Further into development, chondrocytes at the center of epiphyses, undergo hypertrophy and express VEGF. (Gerber et al., 1999; Rivas and Shapiro, 2002). Osteoblasts and osteoclasts

are then recruited to form and resorb bone, which leads to the formation of secondary ossification center. Eventually, entire epiphysis is calcified except at apical ends (Rivas and Shapiro, 2002). At metaphyseal ends, epiphyseal growth plate forms to continually provide proliferating chondrocytes for longitudinal growth of the long bone, until ossifying in adolescent age for humans (Boyce et al., 2002; Shapiro et al., 1977). On the opposite end, an articular cartilage develops covering both ends of a long bone. Articular cartilages do not ossify and remain cartilaginous permanently (Rivas and Shapiro, 2002).

1.2.4 Bone remodeling

After the initial development, bone is continuously resorbed and reformed through a process known as a bone remodeling. The bone remodeling occurs at millions of sites to remove fatigued bones to be replaced with new bone, in an asynchronous manner; this repairing of bones reduce the risk of fracture (Parfitt, 1994). In addition, bone remodeling is stimulated to release calcium and phosphate from the bone for homeostasis, for example, in response to parathyroid hormone (Parfitt, 1976). This process is achieved by a transient multicellular organization of cells referred to as a basic multicellular unit (BMU), which consists of osteoclasts resorbing bone at the leading end followed by osteoblasts depositing new osteoid at the rear end (Hadjidakis and Androulakis, 2006; Ott, 2002).

Each bone remodeling cycle consists of four phases: activation, resorption, reversal and formation (Parfitt, 1994). During the activation phase, osteocytes and osteoblasts localized at the target site initiate remodeling by expressing RANKL. Studies to-date have suggested that a number of factors can trigger activation of bone remodeling, including 1) bone fatigue (*i.e.*

development of bone microfracture) and subsequent osteocyte apoptosis (Verborgt et al., 2000), 2) local factors such as insulin-like growth factor, which are released by osteocytes in response to mechanical stimuli (Bravenboer et al., 2001; Rosen et al., 1994), and 3) systemic factors such as parathyroid hormone (Huang et al., 2004). Release of increased amount of RANKL, but decreased OPG, recruit hematopoietic osteoclast precursors from vasculature and induce differentiation (Kobayashi et al., 2009). During the followed resorption phase, mature osteoclasts resorb fatigued bone, until eventually terminating in response to signal unknown and undergoing apoptosis (Hadjidakis and Androulakis, 2006). Once the bone matrix at the site has been cleared, the reversal phase begins, where cells of unknown lineage are recruited to deposit cement line, a thin, sulfur-rich layer of matrix deficient in mineral (Burr et al., 1988). In the final formation phase, osteoblasts are recruited to deposit osteoid, which eventually calcifies (Hadjidakis and Androulakis, 2006).

The process of bone resorption and formation are coupled, allowing each bone remodeling cycle to result with no net change in bone mass; however, the mechanism underlying this coupling remains unknown. The coupling is postulated to be due to controlled release of growth factors, such as RANKL, to control bone resorption by osteoclasts (Hadjidakis and Androulakis, 2006; Sims and Gooi, 2008). Other studies revealed that osteoclasts directly express soluble and membrane-bound factors to influence osteoblast proliferation and differentiation, implicating that osteoclast-osteoblast communication is bidirectional (Pederson et al., 2008; Walker et al., 2008; Zhao et al., 2006).

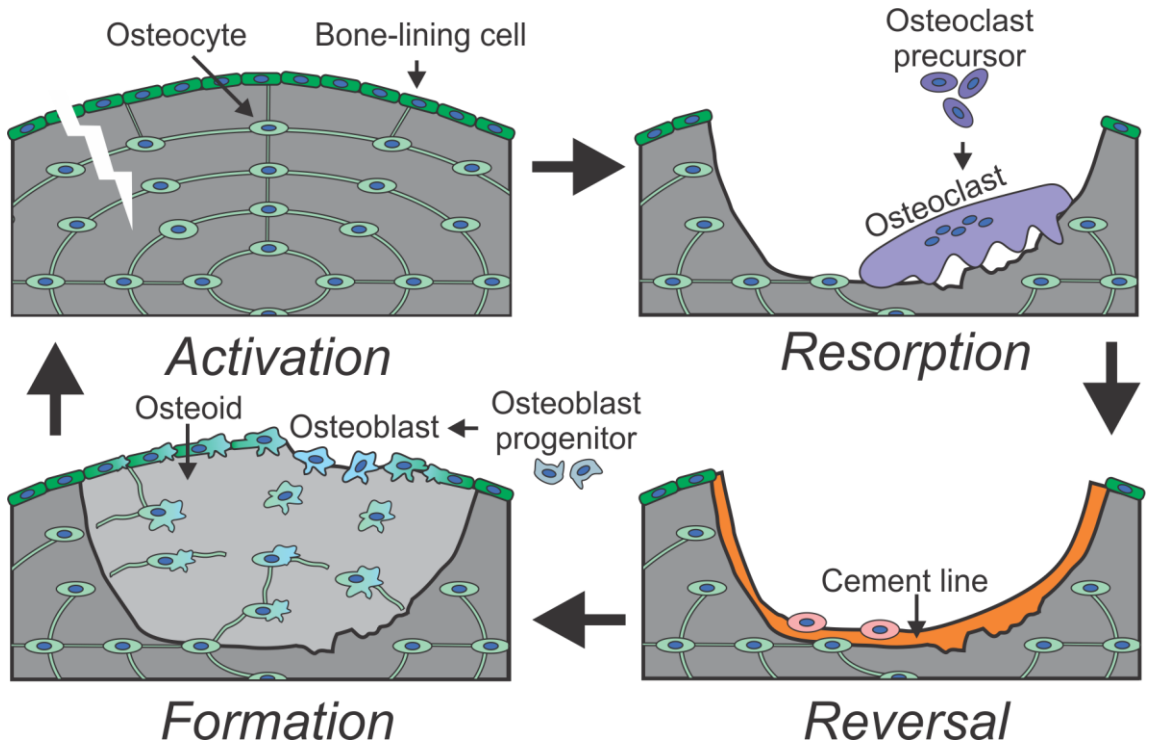


Fig. 1.1. Bone remodeling. Schematic diagram illustrates the process of bone remodeling on trabecular bone. Embedded with mechanosensitive osteocytes and covered by bone-lining cells. *Activation phase*: Excessive mechanical stimulus over time damages bone, forming micro-fractures. In response, osteocytes release pro-osteoclastic RANKL to promote differentiation of osteoclast precursors and survival of mature osteoclasts. *Resorption phase*: Mature osteoclasts 1) demineralize the inorganic component of the matrix by forming an acidic microenvironment and 2) degrade the organic component using proteases and phosphatases. Osteoclasts eventually stop resorbing and undergo apoptosis. *Reversal phase*: Cells of unknown lineage are recruited to deposit a sulfur-rich layer of matrix, called the cement line. *Formation phase*: Osteoblast progenitors are recruited and differentiated into functional osteoblasts. Mature osteoblasts deposit unmineralized type 1 collagen-rich matrix called osteoid, the organic component of the bone. Some osteoblasts undergo terminal differentiation into 1) buried osteocytes or 2) surfaced bone-lining cells; other osteoblasts undergo 3) senescence or 4) apoptosis.

1.2.5 Osteoporosis

Bone formation and resorption are coupled in health individuals and each bone remodeling cycle results in no net change in bone mass. Uncoupled bone formation and resorption can lead to development of pathological conditions; bone formation exceeding bone resorption causes osteopetrosis, a relatively rare condition characterized by increased bone mass and medullary cavity narrowing, usually due to absence or dysfunction of osteoclasts (Stark and Savarirayan, 2009). Oppositely, bone resorption exceeding bone formation causes osteoporosis, a prevalent disease characterized by decreased bone mass, rendering bones susceptible to fracture. Osteoporosis is particularly common in postmenopausal female population due to reduction in systematic level of estrogen, which suppresses osteoclast activity (Eastell et al., 2016).

In a report by Papaioannou et al., three classes of drugs were recommended for the treatment of osteoporosis in Canada: bisphosphonate, denosumab, and parathyroid hormone (PTH) (Papaioannou et al., 2015). Bisphosphonate is an analog of pyrophosphate, with a structure that consists of two phosphates covalently linked to a carbon atom. This center backbone carbon atom has two side chains, which determine pharmacological properties of the drug. Several bisphosphonates have been approved for clinical use, and are broadly classified either as a non-nitrogenous or nitrogenous bisphosphonate – where latter is more pharmacologically efficacious (Fleisch, 1998). Both bisphosphonates work by inhibiting osteoclasts function, but through different mechanisms; non-nitrogenous bisphosphonates are metabolized into toxic ATP analogue (Frith et al., 1997), while nitrogenous bisphosphonates inhibit mevalonate synthesis pathway (van beek et al., 1999). Denosumab is a human monoclonal antibody that binds to RANKL to inhibit RANK-RANKL interaction; like osteoprotegerin. Consequently, osteoclast differentiation is inhibited, ultimately reducing bone

catabolism (Hamdy, 2008). Teriparatide is a 34-amino acid long N-terminal peptide of parathyroid hormone (PTH), which is intermittently administered (*i.e.* daily). Endogenous PTH promotes bone resorption, but the intermittent administration of exogenous PTH induces net bone anabolism (Bodenner et al., 2007). The exact mechanism behind the teriparatide-induced bone anabolism remains unknown. Studies to-date revealed that teriparatide therapy increases the frequency of bone remodeling cycles (Lane et al., 2000) and osteoblast survival is enhanced by the Wnt/Frizzled signaling (Kramer et al., 2010). Until recently, teriparatide was the only FDA-approved bone anabolic agent for treatment of osteoporosis (see below).

1.3 G protein-coupled receptors

1.3.1 Overview of G protein-coupled receptors

G protein-coupled receptors (GPCRs) are seven-transmembrane receptors found in all eukaryotes. They are involved in signal transduction to various stimuli such as proteins, small molecules or electromagnetic radiation (McCudden et al., 2005; Palczewski, 2006). The GPCR superfamily represents the largest superfamily of membrane proteins, with estimated 800 members encoded in the human genome. Based on sequence homology and functional similarity, GPCRs are categorized into one of six families: A, rhodopsin-like; B, secretin-like; C, metabotropic glutamate-like; D, fungal pheromone receptors; E, *Dictyostelium* cAMP receptors; and F, Frizzled/Smoothened (Attwood and Findlay, 1994; Bjarnadottir et al., 2006; Fredriksson et al., 2003). Activation of a GPCR is mediated by interaction between the receptor and an agonist, which may be introduced in an autocrine, paracrine, endocrine, or pharmacological manner (Gilman, 1987; McCudden et al., 2005). Despite the structural and sequential homology among the superfamily of GPCRs, different receptors exhibit affinity to distinct ligands. Ligand binding in family A receptors often occurs at transmembrane loop 3, 5 and 6. Extracellular loops also contribute to such binding, in a receptor dependent manner (Ji et al., 1998). Family B1 receptors, which are targeted by short peptides, also implicate transmembrane loop 3,5 and 6 and extracellular loops, but N-terminal arm as well. For example, activation of parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) by parathyroid hormone (PTH) occurs in a two-step manner. First, PTH (specifically via amino acid residues16-34) binds to N-terminal arm with high affinity and faster kinetics. Then, PTH (via residues 1-14) binds to transmembrane region with lower affinity and slower kinetics (Castro et al., 2005). Similarly, N-

terminal arm was demonstrated to be critical for ligand binding for other family B1 receptors such as calcitonin receptor and glucagon receptor (Stroop et al., 1996; Unson et al., 1996). In a family C GPCR, binding of the ligand is to the N-terminal arm, which is called Venus Flytrap (VFT). Following this interaction, family C GPCRs dimerize to induce signaling (Unson et al., 1996). Thus, ligand binding in different families of GPCR occurs in distinct ways; this and distinctive tissue expression patterns make GPCRs a desirable drug target. Consequently, over 50 percent of marketed drugs are estimated to implicate GPCRs (Schoneberg et al., 2004).

1.3.2 G protein-coupled receptor signaling

Heterotrimeric G proteins are protein trimers of Gα, Gβ and Gγ subunits that mediate GPCR signaling. Heterotrimeric G proteins remain anchored to the plasma membrane, presumably, to allow for rapid interaction with GPCRs (Gilman, 1987); the α subunit is myristiolated or palmitoylated and γ subunit is prenylated (Linder et al., 1993; Wedegaertner et al., 1995). These lipid modifications are thought to anchor Gα and γ subunits to the membrane. Although the Gβ subunit does not undergo such lipid modification, it remains as a heterodimer with Gγ and tends to remain near the membrane as well (Casey, 1994).

The Gα subunit has a nucleotide binding site that determines the activation status of the G protein. Prior to stimulation, Gα is typically bound to GDP and remains in an inactive form. When an agonist activates a GPCR, this GDP is released, and a GTP binds (Hepler and Gilman, 1992; McCudden et al., 2005). GTP-bound G protein then dissociates from the receptor, and breaks apart into a Gα monomer and Gβγ dimer (Wall et al., 1998). The Gα monomer and the Gβγ dimer independently initiate signaling cascades (see below), until GTP is hydrolyzed into

GDP by the Gα subunit; the Gα subunit has a endogenous GTPase catalytic function (Clapham and Neer, 1997; Hepler and Gilman, 1992). Then, Gα-GDP reassociates with Gβγ, returning the G protein to the inactive heterotrimeric state (Gilman, 1987).

The unidirectional and cyclical interaction between G protein and guanidine nucleotide sets the framework to GPCR signaling; the fine-tuning of signaling, however, is mediated by other proteins (Ross, 2008). The dissociation of GDP from Gα protein, and subsequent association with GTP is modulated by guanine nucleotide exchange factors (GEFs). Although the exact mechanisms of GDP release from Gα subunit is not known, it is thought that GPCR activation causes a conformation change in the Gα subunit, to expose the guanidine nucleotide binding site of the Gα subunit (Iiri et al., 1998). Once GDP is released, the Gα subunit transiently exhibits guanidine nucleotide-free state before GTP binding. Gα subunit then binds to GTP rather than GDP because 1) of higher cytosolic concentrations of GTP than GDP (Birnbaumer and Zurita, 2010; Traut, 1994) and 2) stabilization of Gα subunit-GTP complex by Mg^{2+} (Birnbaumer and Zurita, 2010). Although GTP is hydrolyzed by the endogenous catalytic activity of the Gα subunit, intrinsic GTPase activity of Gα subunit is thought to be slow (Ross, 2008). In turn, GTPase activity is accelerated by GTPase-activating proteins (GAPs), such as a protein family of regulator of G protein signaling (RGS). In this regard, the interaction between RGS and the Gα-GTP complex stabilizes the hydrolytic conformation of the Gα subunit, stimulating GTPase function (Tesmer et al., 1997). Another important class of proteins that affect G protein signaling is guanidine nucleotide dissociation inhibitor (GDI), which inhibits dissociation of GDP and thereby prevents subsequent association with GTP. Again, RGS proteins are implicated with GDI function; RGS12 and 14 contain a GoLoco domain, which stabilizes the Gα-GDP complex to prevent spontaneous release of GDP (Kimple et al., 2001;

Traver et al., 2004). RGS proteins are also known to regulate G protein signaling in a manner distinct from the known GAP and GDI activity; for example certain isoforms of RGS have been shown to direct interact with adenylyl cyclase (Sadana and Dessauer, 2009). In this regard, RGS2 has been shown to interact with the C1 domain, first intracellular loop implicated with catalytic activity of adenylyl cyclase. This interaction inhibits cyclic AMP synthesis (Salim et al., 2003). Moreover, some isoforms of RGS have been suggested to interact with MAPK signaling components as a scaffolding protein (Willard et al., 2007), to modulate ion channel function (Schiff et al., 2000), or to regulate transcription (Chatterjee and Fisher, 2002).

Gα-GTP induces signaling in a subtype-dependent manner. Based on sequence homology, Gα subunits are subdivided into one of four protein families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$ (Sunahara et al., 1996). The $G\alpha_s$ family consists of $G\alpha_s$ and $G\alpha_{olf}$ (Downes and Gautam, 1999). Upon activation, members of this Gα family activate adenylyl cyclase, which synthesizes cyclic adenosine 3', 5'-monophosphate (cAMP) using ATP as a substrate (Limbird, 1981). Adenylyl cyclases are membrane-bound proteins with the exception of adenylyl cyclase 10, which is a soluble isoform (Ladilov and Appukuttan, 2014). In addition to activation by $G\alpha_s$, adenylyl cyclases are activated by various effectors and categorized into one of group 1-4 based on it. Briefly, group 1 adenylyl cyclases are inhibited by Gβγ dimer, but stimulated by Ca^{2+}/calmodulin and weakly by PKC. Group 2 are stimulated by Gβγ dimer and group 3 are stimulated by Gβγ dimer, but inhibited by PKA. Group 4 is not affected by Gβγ dimer and is the only the group that exhibits weak activation in response to forskolin (Sadana and Dessauer, 2009).

Free cytosolic cAMP interacts with various effectors. i) cAMP binds to the regulatory subunits of protein kinase A (PKA), causing the release of two catalytic subunits of PKA from a

PKA heterotetramer complex (Knighton et al., 1991). Released PKA catalytic subunit localizes to its target proteins with aid of scaffolding proteins such as A kinase activating protein (AKAP) (Welch et al., 2010). PKA then phosphorylates its target, such as the transcription factor cAMP response element binding protein (CREB), a transcription factor found in the nucleus (Lee and Masson, 1993). ii) cAMP activates exchange protein activated by cAMP (EPAC), a Rap-guanidine nucleotide exchange factor (RapGEF). Activation of EPAC results in GTP association of Rap, which leads to a cascade of MAPK signaling, resulting ultimately in the activation of ERK1/2 (Breckler et al., 2011). iii) cAMP activates cyclic nucleotide-gated (CNG) ion channels. These channels are also activated by cGMP in an isoform-dependent manner; during phototransduction, cGMP, but not cAMP, activates channels to induce inward flow of cations. In contrast, both cAMP and cGMP activate CNG channels during olfaction (Kaupp and Seifert, 2002). iv) cAMP interacts with members of the Popeye domain containing (POPDC) protein family, the function of which is poorly understood (Brand and Schindler, 2017). Thus, activation of $G\alpha_s$ family and subsequent rise in cytosolic cAMP induces signaling through various effectors.

Activation of $G\alpha_s$ simultaneously induces negative-feedback regulation, which occurs at various levels of signaling cascade. Followed by $G\alpha_s$ activation, phosphodiesterases are directly activated by PKA via phosphorylation to degrade cAMP into AMP (Ekholm et al., 1997). Furthermore, PKA upregulates RGS2 expression, which in turn inhibits adenylyl cyclase (Sadana and Dessauer, 2009; Sayasith et al., 2014).

The $G\alpha_i$ protein family is composed of $G\alpha_i$, $G\alpha_o$, $G\alpha_z$, $G\alpha_t$ and $G\alpha_{gust}$ (Downes and Gautam, 1999). Activation of $G\alpha_i$, $G\alpha_o$ and $G\alpha_z$, inhibits group 1, 3 and 4 adenylyl cyclases; aside from this inhibitory role, much regarding the signaling via $G\alpha_i$ protein family is poorly understood (Wong et al., 1991). It is thought that the $G\alpha_i$ signaling is mediated primarily by the

dissociated Gβγ dimer (Birnbaumer, 2007). $G\alpha_t$ and $G\alpha_{gust}$ activate guanylate phosphodiesterase (McLaughlin et al., 1992; Vuong et al., 1984). The $G\alpha_q$ protein family is composed of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$ (Downes and Gautam, 1999). Upon activation, members of this protein family activate phospholipase Cβ (PLCβ), which cleaves membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2), producing cytosolic inositol 1,4,5-trisphosphate (IP_3) and membrane-bound diacylglycerol (DAG) (Berstein et al., 1992). Cytosolic IP_3 activates IP_3-gated Ca^{2+} channels, which release Ca^{2+} from the endoplasmic or sarcoplasmic reticulum, causing a transient elevation of cytosolic free Ca^{2+}. The rise of $[Ca^{2+}]_i$ and increased availability of DAG results in activation of downstream effector proteins, such as calmodulin-dependent kinases/phosphatases and protein kinase C (PKC) (Exton, 1996). The $G\alpha_{12/13}$ protein family is composed of $G\alpha_{12}$ and $G\alpha_{13}$ (Downes and Gautam, 1999). Upon activation, $G\alpha_{13}$, which is competitively inhibited by $G\alpha_{12}$, activates RhoGEF, which exchanges GDP bound to RhoA small GTPase with GTP (Hart et al., 1998). This exchange activates RhoA, which then stimulate cytoskeletal rearrangement (Dhanasekaran and Dermott, 1996).

Prior to G protein activation, the Gβγ dimer increases the affinity of Gα subunit to GDP, thereby stabilizing the inactive conformation of the Gα subunit (Brandt and Ross, 1985). Following GPCR activation and the subsequent release of Gα monomer, Gβ and Gγ subunits remain as a stable dimer and may interact with various effector proteins, in an isoform-dependent manner (Clapham and Neer, 1997). In this regard, activation of the G protein-coupled inwardly-rectifying K^+ channel (GIRK), which causes hyperpolarization, is mediated by βγ dimers that contains β_{1-4}, but not the β_5 isoform (Mirshahi et al., 2002). Likewise, Gβγ dimers can stimulate or inhibit voltage-dependent Ca^{2+} channels (Lotersztajn et al., 1992), adenylyl cyclases (Tang

and Gilman, 1991), phospholipase C, phosphatidylinositol-3-kinase (Stephens et al., 1994) and mitogen-activated protein kinase (Hasson et al., 1994) in a subunit-dependent manner.

Regulation of a ligand-bound GPCR activity is mediated by a family of scaffolding proteins called arrestins. Four arrestin proteins are expressed in mammals: Visual GPCRs (*e.g.* rhodopsin) are regulated by arrestin-1 and 4, while non-visual GPCRs are regulated by arrestin-2 and 3 (Freedman and Lefkowitz, 1996; Luttrell and Lefkowitz, 2002). Historically, arrestin-2 and 3 were first discovered via their interactions with the β-adrenergic receptor; thus arrestin-2 and 3 obtained aliases β-arrestin-1 and 2, respectively (Attramadal et al., 1992; Lohse et al., 1990). In mammals, β-arrestin-1 and 2 are expressed by genes *ARRB1* and *ARRB2*, respectively. Following the activation of a GPCR, a GPCR kinase (GRK) is recruited to phosphorylate the receptor at the C-terminal tail (Bouvier et al., 1988), to increase affinity of the receptor for β-arrestin (Lefkowitz, 1993; Lohse et al., 1992). Then, the β-arrestin is recruited to act as an adaptor to components necessary for clatherin-mediated endocytosis, such as clatherin and assembly protein complex 2 (Gaidarov and Keen, 1999; Goodman et al., 1996). Originally, the internalization of GPCRs was thought to terminate GPCR signaling by impeding G protein association (Goodman et al., 1996). In this regard, *ARRB1*$^{-/-}$ mice exhibit excessive hemodynamic responses to the β-adrenergic receptor agonist isoproterenol (Conner et al., 1997). Similarly, *ARRB2*$^{-/-}$ mice exhibit a prolonged analgesic response to morphine, a potent agonist at the μ opioid receptor (Bohn et al., 1999). The internalized receptor is thought to either 1) be recycled back to the surface (Sibley et al., 1986) or 2) undergo degradation through the ubiquitin-proteasome pathway (Shenoy et al., 2001). More recently, however, studies have suggested that the internalized receptors 3) can continue to signal within vesicles (Calebiro et al., 2010; Rosciglione et al., 2014), prior to recycling or degradation.

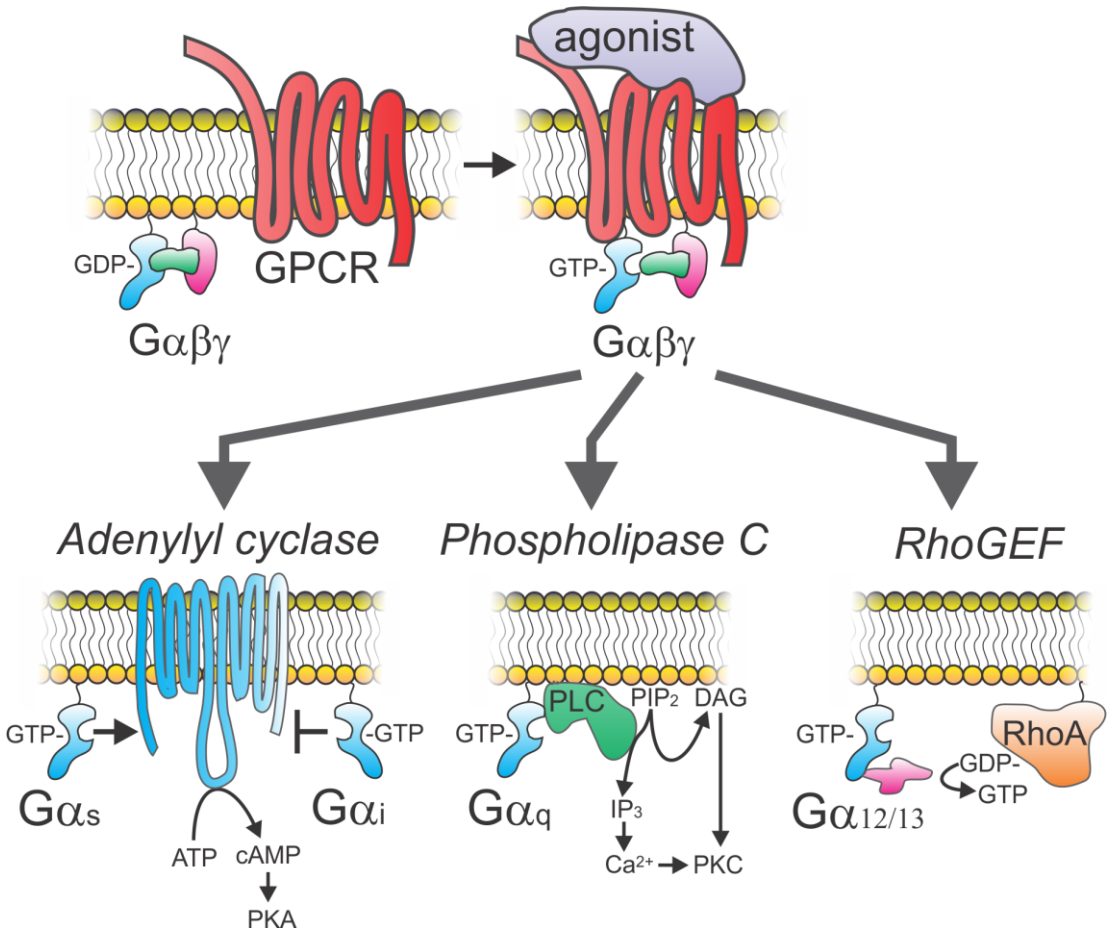


Fig. 1.2. Heterotrimeric G protein-dependent signaling. Agonist binding to a G protein-coupled receptor (GPCR) stimulates the guanine nucleotide exchange factor (GEF) function of the GPCR. Consequently, GDP bound at the α subunit of the G protein (Gα) dissociates and free GTP associates with Gα. The heterotrimeric G protein then dissociates into free Gα and βγ dimer, both of which can induce signaling independently. Gα-mediated signaling is dependent on the protein family of the α subunit: $G\alpha_s$ activates adenylyl cyclase, which, using ATP as a substrate, produces cyclic 3', 5'-adenosine monophosphate (cAMP). Downstream, cAMP activates protein kinase A (PKA). $G\alpha_i$ inhibits adenylyl cyclase. $G\alpha_{q/11}$ activates phospholipase C (PLC), which cleaves membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) into cytosolic inositol 1,4,5-triphosphate (IP_3) and membrane-bound diacylglycerol (DAG). IP_3 releases Ca^{2+} from endoplasmic reticulum. Cytosolic Ca^{2+} and DAG together activate protein kinase C (PKC). $G\alpha_{12/13}$ activates RhoGEF, which exchanges GDP bound at RhoA with GTP.

Later discoveries revealed that the GPCR-associated β-arrestin can signal on its own independently of G proteins (Miller and Lefkowitz, 2001). As β-arrestin is recruited to a GPCR to act as an adaptor for endocytic proteins, Src family tyrosine kinase can be recruited to the β-arrestin. Recruited Src may activate 1) dynamin, the function of which is to cleave the bottleneck-shaped junction between the early endosome and plasma membrane to complete vesicle endocytosis or 2) the small G protein Ras, which can activate EKR1/2 via Raf and MEK (Ahn et al., 1999; Luttrell et al., 1999). In HEK293 cells, overexpression of a mutant β-arrestin that fails to bind to either Src or the receptor results in inhibition of ERK1/2 activation following β_2-adrenergic receptor stimulation (Luttrell et al., 1999). In certain systems, G protein-mediated and β-arrestin-induced MAPK signaling converges at ERK1/2 activation; activation of parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) may result in ERK1/2 phosphorylation by 1) G protein-mediated activation of PKA or PKC and 2) β-arrestin activated MEK (Gesty-Palmer et al., 2006). Similar phenomena have been observed in other GPCRs, including β_2-adrenergic (Shenoy et al., 2006), V2 vasopressin (Ren et al., 2005) and kisspeptin-activated GPR54 (Szereszewski et al., 2010). The consensus among these studies is that ERK1/2 activation via β-arrestin-mediated signaling has a slower onset compared to that triggered via G protein-dependent signaling (Gesty-Palmer et al., 2006; Shenoy et al., 2006).

1.3.3 Allosteric modulation of GPCR signaling

GPCR signaling is initiated by binding of a ligand to the orthosteric site of the receptor. In this regard, binding of an agonist or inverse agonist induces activation or inactivation of the receptor, respectively. In addition to the orthosteric site, GPCRs have distinct interfaces for

ligand interaction known as allosteric sites (Soudijn et al., 2004). Interaction between a ligand and the allosteric site: 1) affects the affinity of the orthosteric site to its ligand; 2) directly activates or inactivates the receptor; and/or 3) alters the efficacy of receptor signaling (Langmead and Christopoulos, 2006). An allosteric modulation influencing the affinity of the orthosteric ligand appears as a change in dissociation constant (K_d) of the orthosteric ligand to the receptor. In this regard, the affinity modulation and consequent change in potency appears as a shift in orthosteric ligand concentration-dependence curve; a positive allosteric modulator (PAM) shifts the curve to the left, whereas a negative allosteric modulator (NAM) shifts the curve to right (Ehlert, 1988). The effects of an allosteric ligand bound to an allosteric site can also resemble those of an orthosteric ligand and influence the intrinsic activity of the receptor directly. In this case, the allosteric ligand may activate the receptor independently of orthosteric ligand binding, a phenomenon known as allosteric agonism (Langmead and Christopoulos, 2006). This phenomenon reflects the ability of an allosteric ligand to change the intrinsic activity of the receptor (Conn et al., 2009). Finally, when intracellular signaling components are affected, the efficacy of the receptor signaling may be influenced (Kenakin, 2005). This effect appears as a change in maximal response and/or shift in the concentration-dependence curve of the orthosteric ligand (Ehlert, 1988).

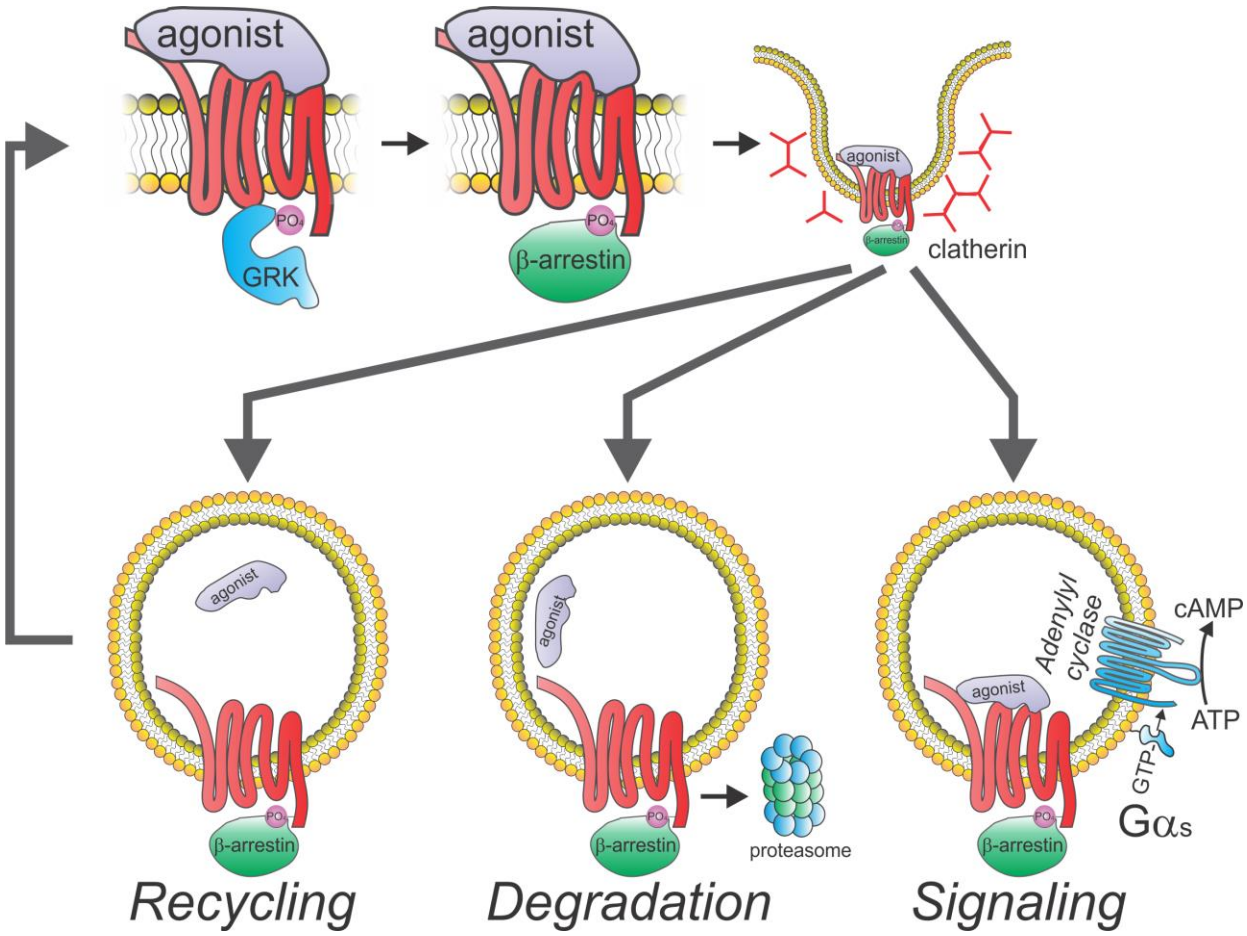


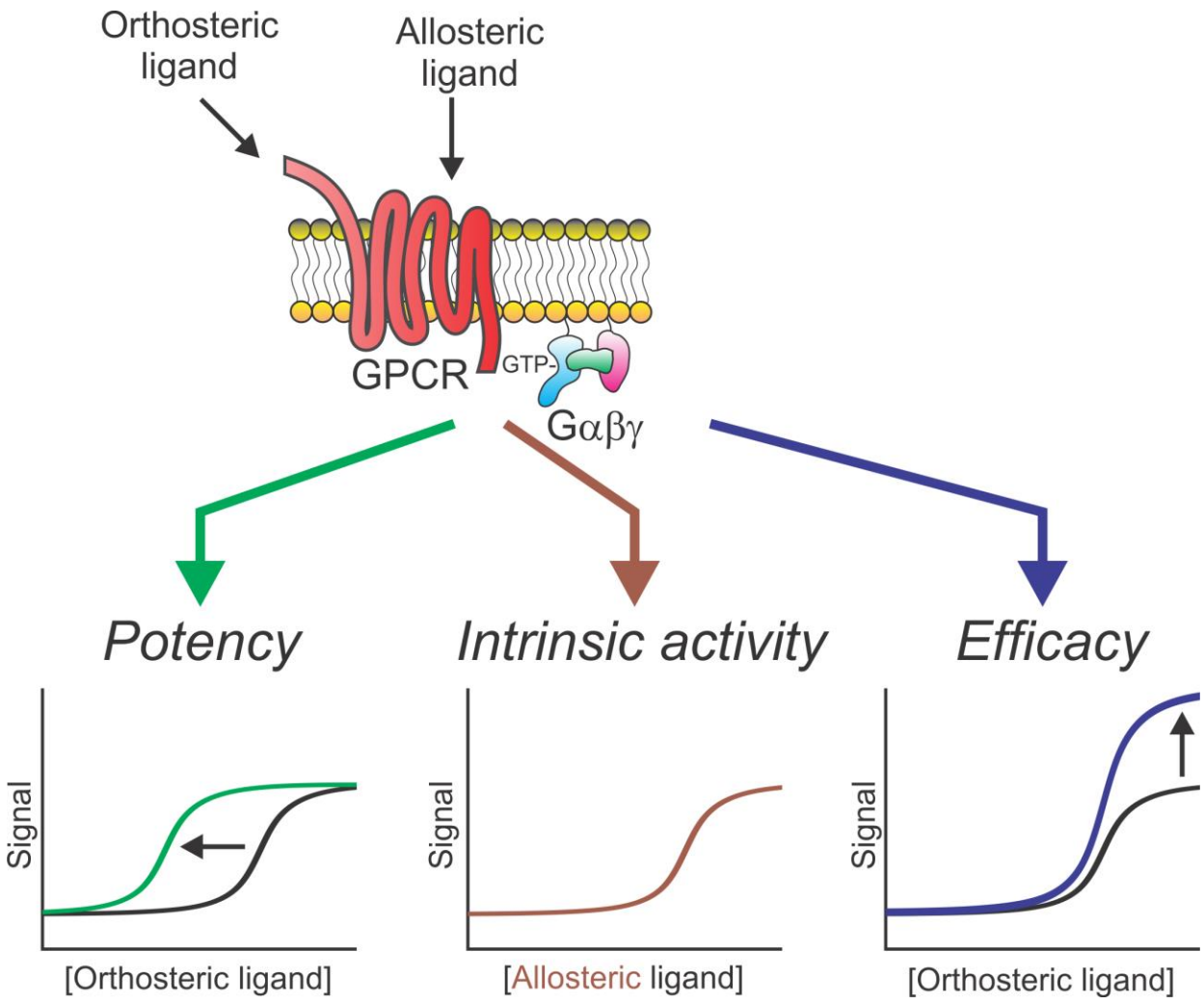
Fig. 1.3. Internalization of G protein-coupled receptor (GPCR). Activation of a GPCR results in recruitment of G protein-coupled receptor kinase (GRK), which phosphorylates the GPCR at the C-terminal tail. Subsequently, β-arrestin is recruited to act as a scaffolding protein for clatherin-mediated internalization. Internalized GPCR is *recycled* back to surface, *degraded* through the ubiquitin-proteasome pathway and/or continue *signaling* within the endosome.

1.3.4 Parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) signaling

Parathyroid hormone/parathyroid hormone-related peptide receptor, also known as parathyroid hormone type 1 receptor (PTH1R), is a family B G protein-coupled receptor (GPCR) that is implicated in skeletal development and calcium/phosphate homeostasis. Activated PTH1R couples to Gs and Gq proteins for signaling via adenylyl cyclase (AC) and phospholipase C (PLC), respectively (Babich et al., 1989; Meltzer et al., 1982; Nissenson and Arnaud, 1979). In addition, PTH1R has been demonstrated to signal through the MAPK pathway via β-arrestin-1 and β-arrestin-2, which also cause receptor internalization (Gesty-Palmer et al., 2006). Few human diseases pertaining to PTH1R mutation have been reported: Blomstrand type chondrodysplasia is a rare autosomal recessive disorder in humans that is caused by inactivating mutation of PTH1R. This neonatally lethal dwarfism is caused by premature chondrocyte hypertrophy and skeletal maturation (Jobert et al., 1998; Young et al., 1993). In contrary, Jansen type metaphyseal chondrodysplasia is a progressive disorder caused by activating mutation of PTH1R. Affected individuals experience various conditions such as wide cranial sutures and shortening of limbs (Schipani et al., 1996).

PTH1R is activated by parathyroid hormone-related peptide (PTHrP) and parathyroid hormone (PTH) (Abou-Samra et al., 1992). PTHrP is an autocrine and paracrine signaling factor that is expressed by many tissue types including cartilage, skin, mammary gland and bone, and is implicated in various physiological functions (Lee et al., 1995). In rodents, initial translation of PTHrP gene renders a 175-amino acid prepropeptide, which undergoes a post-translational modification that 1) removes the signaling peptide and propeptide and 2) cleave the remaining

Fig. 1.4. Allosteric modulation of G protein-coupled receptor (GPCR). An allosteric ligand binds to an allosteric site on a GPCR to modulate signaling in various manner: 1) Allosteric modulation of binding affinity between orthosteric ligand and GPCR (green arrow) results in alteration of *agonist potency*. In an orthosteric ligand-concentration dependence curve, change in agonist potency appears as a left-right shift, with the direction depending on the allosteric ligand. Positive allosteric modulators (PAMs) cause a shift to the left, as illustrated in the schematic figure. 2) The allosteric ligand that alters the *intrinsic activity* of a GPCR can stimulate signaling (brown arrow) in the absence of orthosteric ligand. In the allosteric ligand-concentration dependence curve, this phenomenon appears as an enhanced signaling to increased concentration of allosteric ligand. As such, the ability of allosteric ligand to alter the *intrinsic activity* is also referred to as allosteric agonism. 3) Allosteric modulation of downstream signaling components (blue arrow) results in alteration of *signaling efficacy*. In an orthosteric ligand-concentration dependence curve, shift in *efficacy* is omnidirectional; the concentration-dependence curve can exhibit change in maximal response (up-down shift) or ligand concentration-dependence (left-right shift).


Orthosteric ligand
Allosteric ligand
GPCR
GTP
Gαβγ
Potency
Intrinsic activity
Efficacy
Signal
[Orthosteric ligand]
Signal
[Allosteric ligand]
Signal
[Orthosteric ligand]

139-mer peptide into three secretory forms (Burtis, 1992): PTHrP (1-36), a region with sequence homology to PTH and is implicated with PTH1R activation (Abou-Samra et al., 1992); PTHrP (38-94), a midregion peptide implicated with inhibition of pathological mammary gland growth (Luparello et al., 2001); and PTHrP (107-139), the C-terminal region of the peptide that contains a 5-mer called osteostatin (107-111), which inhibits bone resorption by osteoclasts (Fenton et al., 1991).

Homozygous knockout of PTHrP in mice is postnatally lethal due to asphyxia caused by improper endochondral bone development (Karaplis et al., 1994). During hyaline cartilage development, PTHrP activates PTH1R, which is expressed by slowly proliferating chondrocytes near early hypertrophic cells; late hypertrophic chondrocytes do not express PTH1R. When endochondral development of PTHrP mutant mice embryos was assessed, the proliferating zone of endochondral skeletons was found to be markedly shortened (Lee et al., 1996). In this regard, another study revealed that PTHrP stimulates chondrocyte proliferation while inhibiting hypertrophy to cause spatiotemporal delay of chondrocyte differentiation for longitudinal growth (Vortkamp et al., 1996). In mice with homozygous knockout mutation of PTHrP rescued with a COL2-promoted PTHrP transgene, the endochondral skeleton develops properly, but the mice die within six months due to other complications. Further investigations revealed that tooth eruption failed in these transgenic mice and that PTHrP is crucial for the resorption of alveolar bone by osteoclasts (Kong et al., 1999; Philbrick et al., 1998). With respect to humans, defective tooth eruption is one of the characteristics observed in individuals affected by Blomstrand's chondrodysplasia (Wysolmerski et al., 2001).

PTH is an endocrine factor secreted by chief cells of the parathyroid gland in response to decreased blood calcium concentration. Like PTHrP, the N-terminal region containing the first

34 amino acids of PTH is implicated with activation of PTH1R (Sneddon et al., 2004). Although the remaining residues of PTH are not required for activation of PTH1R, large quantities of C-terminal fragment – that lacks the first 34 amino acids – are found in humans (Dambacher et al., 1979). Later studies demonstrated that the C-terminal fragment is secreted by cells parathyroid gland (Morrissey et al., 1980) or produced by proteolysis of 84-mer hormone (D'Amour et al., 1981). In regards to its function, Selim et al. (2006) reported that these C-terminal fragments are implicated with activation of calcium channels to induce influx of extracellular calcium in osteocytes (Selim et al., 2006). However, the mechanism or receptor implicated in this process remain unelucidated.

In bone, PTH1R is expressed by osteoblasts and osteocytes, but not osteoclasts. PTH1R signaling in osteoblasts or osteocytes has both pro-osteoblastic and pro-osteoclastic effects: PTH1R signaling in osteoblasts promotes their survival and the secretion of RANKL (Bellido et al., 2003; Yasuda et al., 1998). Likewise, PTH1R signaling in osteocytes inhibits secretion of anti-osteogenic sclerostin and stimulates secretion of RANKL (Bellido et al., 2005; Powell et al., 2011). In kidney, PTH1R signaling leads to reduced phosphate reabsorption by cells of the proximal convoluted tubule, but increased calcium reabsorption by cells of distal convoluted tubule (Greger et al., 1977; Shareghi and Stoner, 1978). Furthermore, PTH signaling in kidney increases the expression of 25-hydroxyvitamin D3-1α-hydroxylase, an enzyme that hydroxylates 25-hydroxyvitamin D_3 to produce 1,25-dihydroxyvitamin D_3. This product is the active form of vitamin D that promotes calcium absorption in intestine (Bouillon et al., 2003; Brenza et al., 1998; Miao et al., 2004).

Despite that PTH stimulates osteoclast-dependent bone resorption, intermittent administration of teriparatide – recombinant human PTH (1-34) – induces bone anabolism

(Girotra et al., 2006). Teriparatide was the only marketed drug capable of bone anabolism until abaloparatide was approved by FDA recently. Abaloparatide is a 34-mer PTHrP analog with 76% homology to PTHrP that, like teriparatide, has been demonstrated to reduce the risk of both vertebral and non-vertebral fracture by inducing bone formation – but with reduced risk of hypercalcemia compared to teriparatide (Leder et al., 2015; Miller et al., 2016; Varela et al., 2017). Interestingly, bone anabolic effects of abaloparatide and teriparatide on lumbar spine and femoral neck are similar despite that the prescription dosage for abaloparatide is four times greater than teriparatide (80 *vs.* 20 μg, respectively) (Eli Lilly Inc., 2012; Leder et al., 2015; Radius Health Inc., 2017). In this regard, an *in vitro* study assessing cAMP signalling suggested that abaloparatide favours a conformation of PTH1R that strongly interacts with G protein – denoted R^G conformation – to induce weaker transient cAMP signaling than teriparatide (Hattersley et al., 2016; Vilardaga et al., 2012); though, it is not yet known whether the aforementioned *in vitro* difference accounts for the clinical differences between abaloparatide and teriparatide.

1.4 Nucleotide and G protein-coupled receptor

1.4.1 Nucleotides

Together with carbohydrates and amino acids, nucleic acids are crucial basic building blocks of all known life. All organisms use adenine, cytosine, guanine, thymine and uracil to build a nucleoside – nucleobase bonded to ribose sugar – and a nucleotide – nucleobase bonded to ribose and phosphate(s). Aside from well-established role of nucleotides as 1) building blocks for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) or 2) energy molecules, nucleotides are also known to act as 3) allosteric regulator at various processes (Lu et al., 2014) and 4) primary messengers in the extracellular environment (Burnstock, 2007). Nucleotides are abundant within the cytosolic, but not extracellular environment. Intracellular concentration of ATP is estimated to be in the low millimolar range (*i.e.* 10^{-3} M), though estimates range vary between studies, while other triphosphate nucleotides (*i.e.* CTP, GTP, TTP and UTP) are predicted to be in the high micromolar range (*i.e.* 10^{-4} M) (Traut, 1994). Although the concentration range of extracellular nucleotide is not well known, it is thought that only sparse amounts are present in the absence of stimulus as released nucleotides are readily degraded by extracellular nucleotidases (Yegutkin, 2014).

1.4.2 Extracellular nucleotides

Many cell types release ATP into the extracellular environment in response to various stimuli, such as mechanical stimulus-induced fluid shear or hypoxia (Genetos et al., 2005; Gerasimovskaya et al., 2002; Graff et al., 2000; Orriss et al., 2009). Mechanisms of release

include vesicular exocytosis, channels or ATP transporters (Suadicani et al., 2006; Zhang et al., 2007). Released nucleotides are thought to act as 1) signaling molecules, in autocrine and paracrine processes, or 2) substrates for to energy-dependent processes, briefly prior to degradation by extracellular nucleotidases (Burnstock, 2007; Yegutkin, 2014; Zhu et al., 1997). Signaling in response to extracellular nucleotides is mediated by the P2 receptor superfamily, which consists of P2X and P2Y receptor families. P2X receptor family consists of seven subtypes, P2X1-7, which are ATP-gated cation channels. These homo- or heterotrimeric channels are activated at micromolar concentration of ATP, with exception of P2X7, which requires a millimolar concentration (Aschrafi et al., 2004; Burnstock, 2007; Nicke et al., 1998). P2Y receptor family are family A GPCRs that consist of eight subtypes in mammals: $P2Y_{1,}$ $P2Y_{2,}$ $P2Y_{4,}$ $P2Y_6$ and $P2Y_{11\text{-}14}$. $P2Y_{1,}$ $P2Y_{2,}$ $P2Y_{4,}$ $P2Y_6$ and $P2Y_{11}$ couple to Gq/11 protein; $P2Y_4$ and $P2Y_{12\text{-}14}$ couple to Gi protein; only $P2Y_{11}$ couples to Gs protein; and $P2Y_{1,}$ $P2Y_{2,}$ $P2Y_{4,}$ $P2Y_6$, $P2Y_{11}$ and $P2Y_{14}$ also couple to G12/13 protein. While P2X activation is solely mediated by ATP, P2Y is activated by various nucleotides; both ATP and ADP are endogenous agonists of $P2Y_1$, but ADP has higher affinity than ATP. Likewise, UTP has similar or greater affinity than ATP at $P2Y_{2,}$ $P2Y_4$ and $P2Y_6$ (Burnstock, 2007).

In bone, mechanical stimuli drive remodeling in favour of osteogenesis, leading to increased bone mass (Forwood, 2001; Robling et al., 2002) and accumulated evidence suggests that osteoblasts release ATP in response to mechanical stimuli, such as fluid shear (Genetos et al., 2005). Accordingly, P2 signaling by released ATP has been suggested to facilitate mechanotransduction to induce bone anabolism (Dixon and Sims, 2000; Romanello et al., 2001). However, the consequence of bone formation and resorption to P2 signalling remain debated. Xing et al. (2014) suggested that $P2Y_2$ is critical to bone anabolic response to mechanical

stimulus, as the study observed that 1) mice with homozygous knockout mutation of $P2Y_2$ exhibited reduced bone formation to fluid shear. The study further demonstrated that 2) primary osteoblasts isolated from $P2Y_2$ knockout mice or control mice released ATP at similar levels in response to fluid flow, indicating that $P2Y_2$ is not involved in ATP release (Xing et al., 2014). In contrast, Orriss et al. (2017) reported that 1) the $P2Y_2$ activation induces ATP release, which 2) does not influence bone formation, but 3) enhances bone resorption, in a P2X7-dependent manner (Orriss et al., 2017); interestingly, the role of P2X7 activation in bone formation and resorption also remain debated (Noronha-Matos et al., 2014; Orriss et al., 2013; Panupinthu et al., 2008). Regarding the discrepancies between reports, Orriss et al. (2017) suggested that strain difference of studied mice may be responsible for the observed inconsistencies between reports.

In addition to the ability to induce P2 receptor activation and subsequent signaling, ATP is also implicated in energy-dependent processes in the extracellular environment. In this regard, evidence suggests that many protein kinases that use ATP as a substrate, such as protein kinase A and C, are found in the extracellular environment – as ectokinases. Ectokinases appear to be implicated in various physiological as well as pathological processes (Hogan et al., 1995; Kondrashin et al., 1999; Yalak et al., 2014; Zhu et al., 1997). In addition to ectokinases, ATP is targeted by extracellular phosphatases, such as ectonucleotidase, inorganic pyrophosphatase and alkaline phosphatase, and is eventually metabolized to adenosine (Yegutkin, 2014). Extracellular adenosine can 1) signal through P1 adenosine receptors (Burnstock, 2007) or 2) bet taken up by cells through nucleoside transporters (Griffiths et al., 1997). Nucleotide metabolism also releases inorganic phosphate, which contributes to calcification of bone (Farrow and White, 2010).

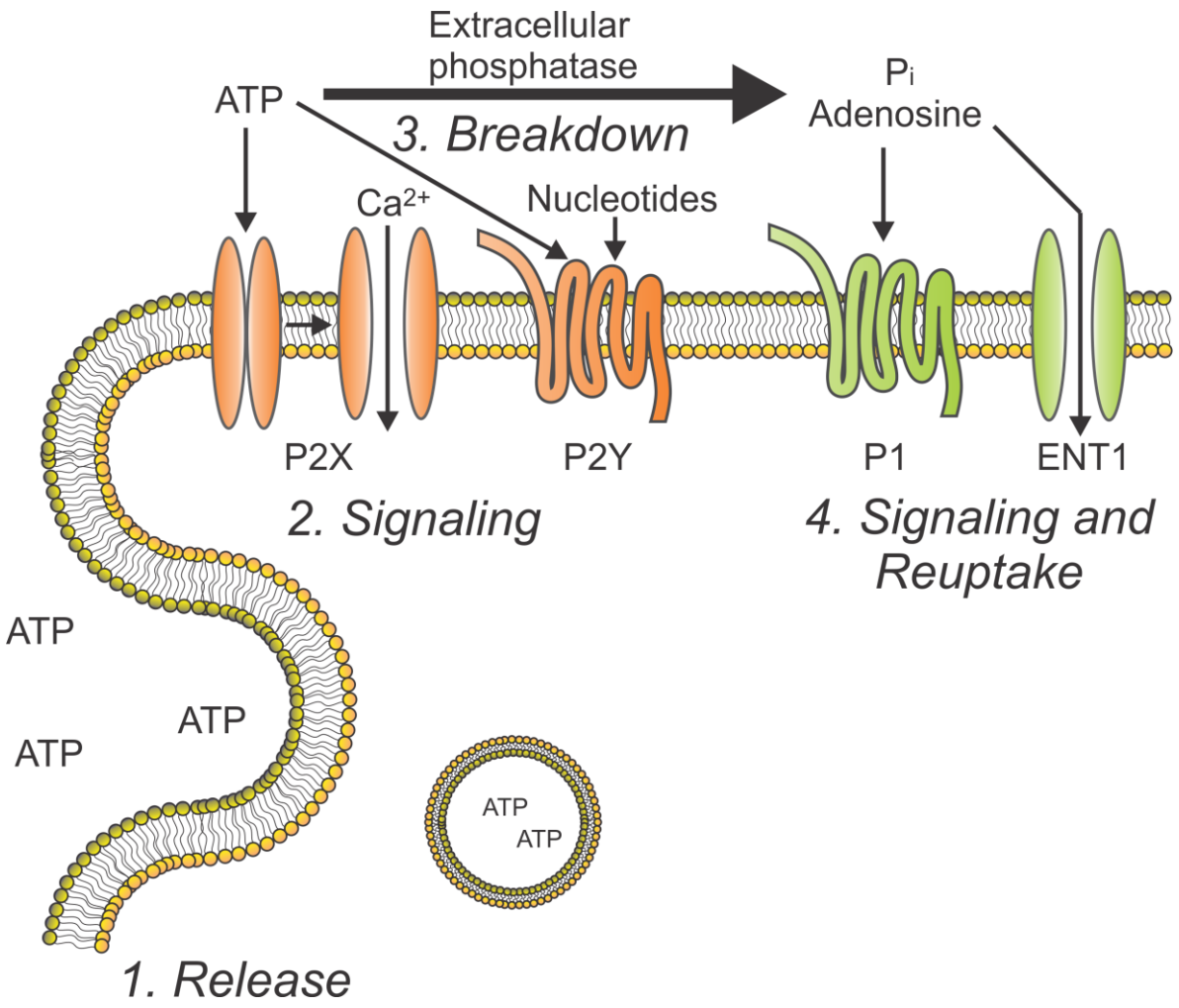


Fig. 1.5. Extracellular adenosine 5'-triphosphate (ATP). In response to various stimuli, cells 1) *release* ATP to the extracellular environment. Extracellular ATP acts as a 2) *signaling* molecule in an autocrine or paracrine manner, by activating P2X cation channel or P2Y G protein-coupled receptors. While P2X activation is solely mediated by ATP, P2Y can be activated by other nucleotides such as ADP, UTP or UDP. Extracellular ATP is short-lived, as ectonucleotidases target it for 3) *degradation*; ATP is metabolized into adenosine and inorganic phosphate (P_i). Adenosine can further 4) *signal* through P1 receptors or be *taken up* by cells via nucleoside transporters such as equilibrative nucleoside transporter 1 (ENT1).

1.4.3 Nucleotides act as allosteric modulators

The ability of ATP to allosterically regulate intracellular processes is documented in various physiological processes. Phosphofructokinase, a key enzyme in glycolysis that phosphorylates fructose 6-phosphate into fructose 1,6-bisphosphate, uses ATP as a substrate for kinase function. While so, ATP also acts as a negative allosteric modulator (NAM) to phosphofructokinase, inhibiting downstream synthesis of ATP (Babul, 1978; Usenik and Legisa, 2010). Similarly, ATP acts as a NAM to glycogen phosphorylase to inhibit glycogen mobilization to 1) reduce blood glucose level and 2) inhibit downstream ATP synthesis (Rath et al., 2000). Likewise, ATP has been reported to allosterically modulate numerous proteins including dnaA, uridine monophosphate kinase and ribonucleotide reductase (Eriksson et al., 1997; Erzberger et al., 2006; Meier et al., 2008). Allostery by extracellular ATP has been documented only recently; Liu and Wang (2014) reported that extracellular ATP potentiates ionotropic γ-aminobutyric acid (GABA) type A receptor ($GABA_AR$) activation by GABA. The study proposed that the potentiation was mediated by allosteric modulation by ATP, and that neither P2 receptors nor ectokinases were implicated. Notably, potentiation occurred in the presence of ATP at millimolar concentrations (Liu and Wang, 2014). Allosteric modulation by extracellular ATP has not been documented for any other receptor.

1.4.4 Nucleotides influence GPCR signaling

Numerous studies been reported that extracellular nucleotides, through P2 receptor activation, influence signaling by various GPCRs. For example, $P2Y_{12}$-induced G_i signaling and subsequent PI3K activation has been shown to be required for platelet aggregation in response to

thromboxane A_2 receptor activation (Dangelmaier et al., 2001; Paul et al., 1999). P2 activation has also been shown to synergistically crosstalk with other GPCRs, to enhance signaling. In this regard, both unidirectional (*i.e.* P2 receptor influencing signaling to another GPCR) and bidirectional (*i.e.* P2 receptor potentiating signaling by another GPCR and vice versa) crosstalk have been reported. Grol et al. (2016) reported that P2X7 activation results in downregulation of GSK3β, which inhibits Wnt-induced β-catenin nuclear translocation (Grol et al., 2016). Kapoor and Sladek (2000) examined *ex vivo* brain specimens containing supraoptic neurons, and demonstrated that P2 activation results in amplified release of vasopressin in response to α-adrenergic receptor activation (Kapoor and Sladek, 2000). A following study that monitored signaling *in vitro* revealed that $P2Y_1$ activation results in enlargement of a calcium transient induced by α-adrenergic receptor activation, and suggested that the amplified signaling is responsible for observed enhancement of vasopressin release (Espallergues et al., 2007). Similar amplification of calcium transient by nucleotides has been reported for IL-8-induced CXCR2 activation (Werry et al., 2002), bradykinin-induced bradykinin receptor activation and PTH-induced PTH1R activation (Kaplan et al., 1995). With respect to PTH1R, Kaplan et al. (1995) demonstrated that agonists at P2Y receptor potentiated the PTH-induced calcium transient, but not cAMP accumulation (Kaplan et al., 1995). Interestingly, P2Y-mediated potentiation of calcium release to PTH1R activation has also been shown to be bidirectional and PTH1R activation also potentiates calcium release to P2Y activation (Buckley et al., 2001). The ability of extracellular nucleotides to influence signaling to another GPCR independently of P2 receptor activation has not been reported previously.

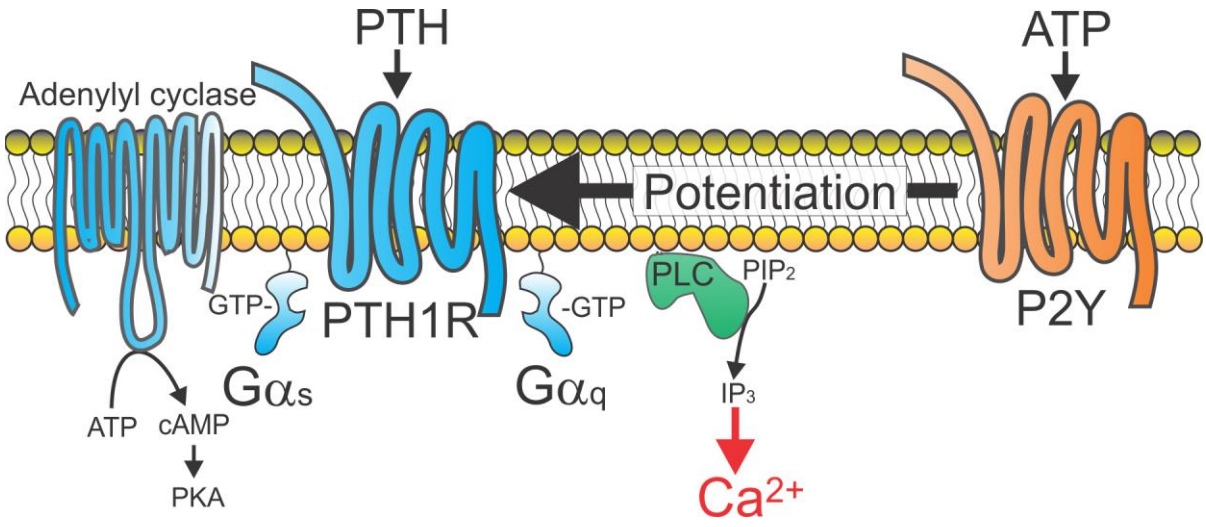


Fig. 1.6. P2Y-mediated potentiation of parathyroid hormone receptor 1 (PTH1R) signaling. Parathyroid hormone (PTH) is an endocrine factor that activates PTH1R, which is a G protein-coupled receptor that signals through $G\alpha_s$/adenylyl cyclase and $G\alpha_q$/phospholipase C (PLC). Consequently, PTH1R activation results in rise in cytosolic cAMP and Ca^{2+} level. Activation of P2Y results in potentiation of PTH-induced PTH1R signaling, which is characterized by enhanced rise in Ca^{2+}, but not cAMP levels.

1.5 Rationale, hypothesis and objectives

Rationale – Extracellular nucleotides are signaling molecules that act through P2 nucleotide receptors (Burnstock, 2007). Previously, low concentrations of nucleotides were shown to act via high affinity P2 receptors to potentiate PTH-induced calcium, but not cAMP signaling (Kaplan et al., 1995). Recently, high concentrations of extracellular ATP were shown to potentiate signaling by other receptors via low-affinity P2X7 receptor-dependent and independent mechanisms (Grol et al., 2016; Liu and Wang, 2014).

Hypothesis – We hypothesize that high concentrations of extracellular ATP will potentiate PTH1R signaling, in a manner distinct from the previously reported effect.

Specific Objective 1 – Investigate the effect of millimolar concentrations of extracellular ATP on PTH1R agonist potency.

Specific Objective 2 – Determine whether other extracellular nucleotides can mimic the effect of ATP.

Specific Objective 3 – Determine whether the effect of ATP on PTH1R signaling extends to the transcriptional level.

Specific Objective 4 – Investigate the mechanism underlying the effect of ATP on PTH1R signaling.

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CHAPTER TWO

EXTRACELLULAR NUCLEOTIDES ENHANCE AGONIST POTENCY AT THE PARATHYROID HORMONE 1 RECEPTOR[1]

[1] This chapter was modified from: Kim, B.H., A. Pereverzev, S. Zhu, A.O.M. Tong, S.J. Dixon, and P. Chidiac. 2018. Extracellular nucleotides enhance agonist potency at the parathyroid hormone 1 receptor. *Cell Signal*. 46:103-112 with permission (Appendix A).

2.1 Introduction

Structural integrity of bone is maintained by the process of bone remodeling, which involves resorption of pre-existing bone by osteoclasts, followed by synthesis of new bone by osteoblasts. Impaired bone remodeling can lead to development of diseases such as osteoporosis, a condition characterized by reduced bone mass and increased risk of fracture (Langdahl et al., 2016). Mechanical loading from physical exercise drives bone remodeling in favor of osteogenesis, leading to increases in bone mass (Klein-Nulend et al., 2012). At the cellular level, mechanical loading causes osteoblasts and other cell types to release ATP into the surrounding extracellular fluid (Burnstock and Knight, 2017; Romanello et al., 2001). Once released into the extracellular milieu, ATP can serve as a substrate for ectokinases (Yalak et al., 2014) or as an agonist at P2 purinergic receptors (Burnstock, 2007).

Parathyroid hormone (PTH) is an important regulator of systemic calcium homeostasis and bone remodeling. PTH activates the PTH/PTH-related peptide (PTHrP) receptor (PTH1R), a family B G protein-coupled receptors (GPCRs). PTH1R couples to: i) Gs to stimulate adenylyl cyclase activity, which elevates levels of cytosolic cyclic AMP (cAMP); ii) Gq to stimulate phospholipase Cβ activity, which leads to release of calcium from intracellular stores and activation of protein kinase C; and iii) β-arrestin to induce internalization of the receptor and signaling via the MAPK/ERK pathway (Cheloha et al., 2015). Increased cytosolic cAMP and/or calcium leads to activation of cAMP response element binding protein (CREB) (Shaywitz and Greenberg, 1999). In bone, activation of PTH1R leads directly to the proliferation and differentiation of osteoblasts (Datta et al., 2007; Isogai et al., 1996), and indirectly to the differentiation of osteoclasts by regulating expression of RANKL and osteoprotegerin (Huang et

al., 2004).

PTH appears to promote the mechanosensitivity of bone *in vivo* (Chow et al., 1998; Gardner et al., 2007); as well, mechanical loading enhances the anabolic effects of intermittent PTH (Sugiyama et al., 2008). The mechanism behind these phenomena is poorly understood. ATP – known to be released in response to mechanical loading – can modulate PTH1R signaling (Kaplan et al., 1995; Short and Taylor, 2000; Sistare et al., 1995). At micromolar concentrations, extracellular nucleotides potentiate the elevation of cytosolic calcium (but not cAMP) induced by PTH, a process that is dependent on activation of high affinity P2 nucleotide receptors (Kaplan et al., 1995). Extracellular ATP can occur at even higher concentrations *in vivo*, where it activates P2X7 – a relatively low affinity nucleotide receptor (Ke et al., 2003). However, the effects of such high concentrations of ATP on PTH1R signaling have not been addressed previously.

Here we show that, at millimolar concentrations, extracellular ATP enhances PTH-induced adenylyl cyclase activation, which is characterized by a substantial shift in measured potency. ATP also potentiates PTH-induced recruitment of β-arrestin-1 to PTH1R, a process independent of G proteins. As well, PTH-induced activation of CREB is enhanced by ATP. Mechanistically, these phenomena were found to be independent of P2 receptors, including P2X7. Furthermore, other nucleotides – such as CMP and the poorly hydrolysable ATP analog AMP-PNP – mimicked the effect of ATP, establishing that potentiation does not require hydrolysis of a high-energy phosphoanhydride bond. Taken together, these data suggest that ATP enhances signaling through a previously unrecognized allosteric interaction with PTH1R or a closely associated protein.

2.2 Materials and methods

2.2.1 Materials and solutions

α-Minimum essential medium (α-MEM), heat-inactivated fetal bovine serum (FBS), antibiotic solution (10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B), trypsin solution, Dulbecco's phosphate-buffered saline (DPBS), Dulbecco's modified Eagle medium (high glucose) (DMEM), CO_2 independent medium, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), minimum essential medium (MEM; without bicarbonate, and with or without phenol red), and indo-1-acetoxymethyl ester (indo-1-AM) were obtained from Thermo Fisher Scientific (Waltham, MA). X-tremeGENE9 was from Roche Diagnostics (Laval, QC, Canada). GloSensor™ cAMP Reagent, 5× Passive Lysis Buffer, and Bright-Glo™ Luciferase Assay System Reagents were obtained from Promega (Madison, WI). Bovine albumin (BSA), Fraction V was obtained from MP Biomedicals (Solon, OH). Adenosine 5′-triphosphate (ATP) disodium salt hydrate; adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP) lithium salt hydrate; adenosine 5′-diphosphate (ADP) disodium salt; guanosine 5′-triphosphate (GTP) sodium salt hydrate; uridine 5′-triphosphate (UTP) trisodium salt hydrate; cytidine 5′-triphosphate (CTP) disodium salt; cytidine 5′-monophosphate (CMP) disodium salt; 3-isobutyl-1 methylxanthine (IBMX); 4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one (Ro 20-1724); and suramin sodium salt were obtained from Sigma-Aldrich (St. Louis, MO). A438079 hydrochloride and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) tetrasodium salt were obtained from Tocris Bioscience (Bristol, UK). D-luciferin sodium salt was obtained from Gold Biotechnology (St. Louis, MO). Ionomycin was from Enzo Life Sciences (Farmingdale, NY). Rat PTH (1-34) and PTHrP (1-34) were purchased from Bachem (Bubendorf,

Switzerland). Rat parathyroid hormone (1-14) was synthesized using the chemical protein synthesis service from GenScript Biotech Corporation (Piscataway, NJ). RAMP expression plasmids (pcDNA3-hRAMP1, pcDNA3-hRAMP2 and pcDNA3-hRAMP3) were generously provided by Dr. Patrick Sexton (Monash Institute of Pharmaceutical Sciences, Melbourne, Australia). Nucleotides were dissolved and diluted in divalent cation-free buffer: 140 mM NaCl, 5 mM KCl, 20 mM HEPES, and 10 mM glucose; pH = 7.3 ± 0.02, 290 ± 5 mOsmol/L (Veh_2). Peptides were dissolved and diluted in DPBS, supplemented with 0.1% BSA (Veh_1). Na^+ solution for fluorescence measurement of cytosolic free Ca^{2+} concentration consisted of 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES, adjusted to pH 7.40 ± 0.02 and 290 ± 5 mOsmol/L.

2.2.2 Cells and culture

UMR-106 rat osteoblast-like cell line (Partridge et al., 1983) and MC3T3-E1 murine osteoblast-like cell line (subclone 4) (Wang et al., 1999) were obtained from the American Type Culture Collection (Rockville, MD); the sex of neither cell line is known (Shah et al., 2014). MC3T3-E1 and UMR-106 cells endogenously express PTH1R and downstream signaling components including adenylyl cyclase and CREB (Forrest et al., 1985; Hakeda et al., 1987; Pearman et al., 1996; Sakamoto et al., 1998). UMR-106 and MC3T3-E1 cells were subcultured twice weekly and maintained at 37°C and 5% CO_2 in α-MEM supplemented with 10% FBS and antibiotic-antimycotic solution (final concentrations: penicillin 100 units/mL; streptomycin 100 μg/mL; and amphotericin B 0.25 μg/mL). For the PTH1R-β-arrestin interaction assay, we used the HEK293H female (Shah et al., 2014) human embryonic kidney cell line (Gibco 293-H cells from

Thermo Fisher Scientific). HEK293H cells were subcultured twice weekly and maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS and antibiotic-antimycotic solution. Cells were used for experimentations at passages below 25.

2.2.3 Transfections

All transfections were performed using X-tremeGENE 9 Reagent according to the manufacturer's protocol with a modification. Briefly, we prepared a DNA transfection complex consisting of DMEM, X-tremeGENE 9 Reagent, and plasmid vector. Next, a cell suspension was prepared by trypsinization followed by resuspension in fresh medium, and DNA transfection complex was added directly to the suspension. After mixing, the cell suspension was plated into multi-well plates as indicated for each experiment.

2.2.4 Live cell cAMP measurement

Cytosolic cAMP levels in live cells were monitored using GloSensor™ cAMP assay according to the manufacturer's protocol. For UMR-106 cells, samples were transfected with pGloSensor™-22F cAMP plasmid (Promega) and the cell suspension was seeded on white clear-bottom (Corning) or white solid-bottom (Greiner Bio-One, Monroe, NC) 96-well plates at a seeding density of 5.0×10^4 cells/well (1.5×10^5 cells/cm^2). The pGloSensor™-22F cAMP plasmid encodes a chimeric protein in which the cAMP-binding domain of protein kinase A is placed between the C- and N-termini of firefly luciferase fragments. The binding of cAMP induces a conformational change that greatly increases luminescence activity (Fan et al., 2008).

After 24 h incubation at 37°C/5% CO_2, cells were placed in fresh MEM (without phenol red; supplemented with 2 mM D-luciferin, 20 mM HEPES and 0.1% BSA; pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L) and were incubated for 2 h at room temperature. Where indicated, cells were treated with IBMX (500 µM) and were further incubated for 20 min at room temperature. Next, cells were stimulated with agonists (time 0) and the emitted luminescence was measured using LMax^{TM}II^{384} plate reader (Molecular Devices) with 1 s integration time at 1.5 min intervals for total of 45 min.

For MC3T3-E1 cells, samples were transfected with pGloSensor™-22F cAMP plasmid and seeded on white clear-bottom 96-well plates with seeding density of 5.0×10^4 cells/well (1.5×10^5 cells/cm^2). After 24-hour incubation at 37°C/5% CO_2, cells were placed in CO_2-independent medium (supplemented with 10% FBS and 0.6 mg/mL of GloSensor™ cAMP Reagent) and were incubated for two hours at room temperature. Next, cells were treated with phosphodiesterase inhibitors Ro 20-1724 (200 µM) and IBMX (500 µM) and were further incubated for 20 min at room temperature, until stimulation with agonists.

For HEK293H cells, samples were co-transfected with two plasmids – pcDNA3.1(+)-hPTH1R (cDNA Resource Center, Bloomsburg University, Bloomsburg, PA) encoding human PTH1R and GloSensor™-22F cAMP biosensor plasmid – and seeded on white clear-bottom 96-well plates at a density of 5.0×10^4 cells/well (1.5×10^5 cells/cm^2). After 24 h incubation at 37°C/5% CO_2, cells were placed in fresh MEM (without phenol red, supplemented with 2 mM D-luciferin, 20 mM HEPES and 0.1% BSA; pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L) and were incubated for two hours at room temperature. Next, cells were treated with IBMX (500 µM) and were further incubated for 20 min at room temperature, until stimulation with agonists.

2.2.5 Immunoassay of cellular cAMP

As an alternative to the GloSensor™ method, cellular cAMP content was measured using cAMP-Screen™ Cyclic AMP Immunoassay System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol (Chiulli et al., 2000). UMR-106 cells were plated on a white clear-bottom 96-well plate (Corning) at a density of 5.0×10^4 cells/well (1.5×10^5 cells/cm^2). After 24 h incubation at 37°C, 5% CO_2, cells were placed in fresh MEM (without phenol red; supplemented with 20 mM HEPES and 0.1% BSA; pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L) and were incubated for 2 h at room temperature. Next, cells were treated with IBMX (500 µM) and further incubated for 20 min at room temperature. Cells were then treated with PTH and/or ATP for 10 min and then lysed. Lysates, cAMP standards, and cAMP-alkaline phosphatase (AP) conjugate were added to the 96-well assay plate pre-coated with goat anti-rabbit IgG. Next, rabbit anti-cAMP antibody was added to each well and incubated for 1 h. Wells were then washed and CSPD/Sapphire-II™ RTU substrate/enhancer solution was added. After 30 min, luminescence was measured using an LMax™II^{384} plate reader with 1 s integration time.

2.2.6 Fluorescence measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)

This procedure was modified from Grol *et al.* (Grol et al., 2012). UMR-106 cells were loaded with indo-1-AM (3 µM) for 30 min at 5% CO_2 and 37°C. Next, cells were washed using DPBS and lifted using trypsin-EDTA solution. Cells were sedimented and resuspended in MEM (with phenol red, and supplemented with 20 mM HEPES). For determinations, an aliquot of cell suspension was sedimented and resuspended in 2 mL of Na^+ solution in a fluorometric cuvette with continuous stirring at 35°C. Test substances were added directly to the cuvette. $[Ca^{2+}]_i$ was

measured using RF-M2004 multi-wavelength spectrofluorimeter and FelixGX software (Horiba, Edison, NJ) at 355 nm excitation. Emission intensities at 405 and 485 nm were used to determine the ratio R, which then allowed $[Ca^{2+}]_i$ to be calculated. Calcium transients were quantified as area under the $[Ca^{2+}]_i$ curve. Basal levels, which were determined as the average $[Ca^{2+}]$ 6 to 14 s prior to exposure to the agonist, were subtracted.

2.2.7 Live cell β-arrestin-1-PTH1R interaction assay

Agonist-promoted binding of β-arrestin-1 to PTH1R was assessed using a luminescent protein complementation assay (Misawa et al., 2010). The plasmid PtGRN415-ARRB1 in pcDNA3.1/myc-His B (encoding N-terminal click beetle luciferase (1-415)-β-arrestin-1 chimeric protein) was generously provided by Dr. Takeaki Ozawa (University of Tokyo, Japan). The second plasmid, hPTH1R-linker20-PtGRC394 in pcDNA3.1(+) (encoding human PTH1R-C-terminal click beetle luciferase (394-542) chimeric protein) was synthesized as follows. An insert, consisting of a C-terminal fragment of human PTH1R-linker20-PtGRC394, was custom synthesized using gBlocks® Gene Fragments service offered by Integrated DNA Technologies (Coralville, IA). The insert was then cloned into the pcDNA3.1(+)-hPTH1R vector using in-frame XhoI-ApaI ligation. Cells co-transfected with both plasmids constitutively express the two chimeric proteins mentioned above. In response to a suitable stimulus, N-terminal luciferase-β-arrestin-1 is recruited to PTH1R-C-terminal luciferase. When in proximity, complementation of the luciferase fragments forms functional luciferase.

HEK293H cells were transfected with the two plasmids (1:1 M ratio) in suspension and the cell suspension was plated on a white clear-bottom 96-well plate (Corning) at a seeding

density of 5×10^4 cells/well (1.5×10^5 cells/cm^2). After 24 h incubation at 37°C, 5% CO_2, cells were placed in fresh MEM (without phenol red; supplemented with 3.2 mM D-luciferin, 20 mM HEPES, and 0.1% BSA; pH = 7.20 ± 0.02; 300 ± 5 mOsmol/L) and were incubated for 1 h at 37°C. Next, cells were stimulated with agonists (time 0) and luminescence was measured using an LMax^{TM}II^{384} plate reader, at 37°C with 2 s integration time at 2 min intervals for total of 80 min.

2.2.8 CRE-reporter luciferase assay

The cAMP response element (CRE)-luciferase reporter plasmid pCRE-TA-Luc was generously provided by Dr. T. Michael Underhill (University of British Columbia, Vancouver, Canada). This plasmid contains 3 CRE sites, which enhance luciferase expression in the presence of activated CRE binding protein (CREB) (Weston et al., 2002). UMR-106 cells were transfected with CRE-luciferase reporter plasmid in suspension and the cell suspension was plated on 48-well cell culture plate (Falcon) at a density of 3×10^4 cells/cm^2. Cells were then incubated for 24 h at 37°C, 5% CO_2; where indicated, serum was removed and cells were incubated for an additional 24 h. Next, cells were treated with agonists (time 0) for the indicated periods. Cells were then incubated in 65 µL 1× Passive Lysis Buffer at room temperature for 30 min for lysate collection. CREB activity was quantified by assessing luminescence; 15 µL lysate was mixed with 15 µL Bright-Glo™ Luciferase Reagent in white solid-bottom 96-well plate (Greiner Bio-One). Then, luminescence was measured using an LMax^{TM}II^{384} plate reader with 2 s integration time.

2.2.9 Data analyses and statistics

Data shown are means ± SEM. Differences between two groups were analyzed using Student's *t*-test. Differences among three or more groups were analyzed using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. Data obtained in the live cell cAMP and β-arrestin-1-PTH1R interaction assays were in the form of response *versus* time curves. For each time-course curve, we calculated a series of average slopes beginning at every point using the next 5 consecutive data points; we then selected the maximal slope, which was normalized as a fraction of the greatest slope within the experiment, and the resultant values were plotted as a function of agonist or extracellular nucleotide concentration. This procedure is illustrated in Fig. 2.2. Concentration-response data were fitted using GraphPad Prism 5 software (La Jolla, CA) to a 3-parameter sigmoidal equation (fixing Hill slope to 1 and varying minimum signal, EC_{50} and maximum signal) using simultaneous nonlinear regression analysis of multiple data sets. The *F*-statistic (calculated using the extra sum-of-squares *F*-test) was used to assess the effect of extracellular nucleotides on EC_{50}, by constraining this parameter to be the same between data sets acquired with and without extracellular nucleotide. Differences were accepted as statistically significant at $p < 0.05$.

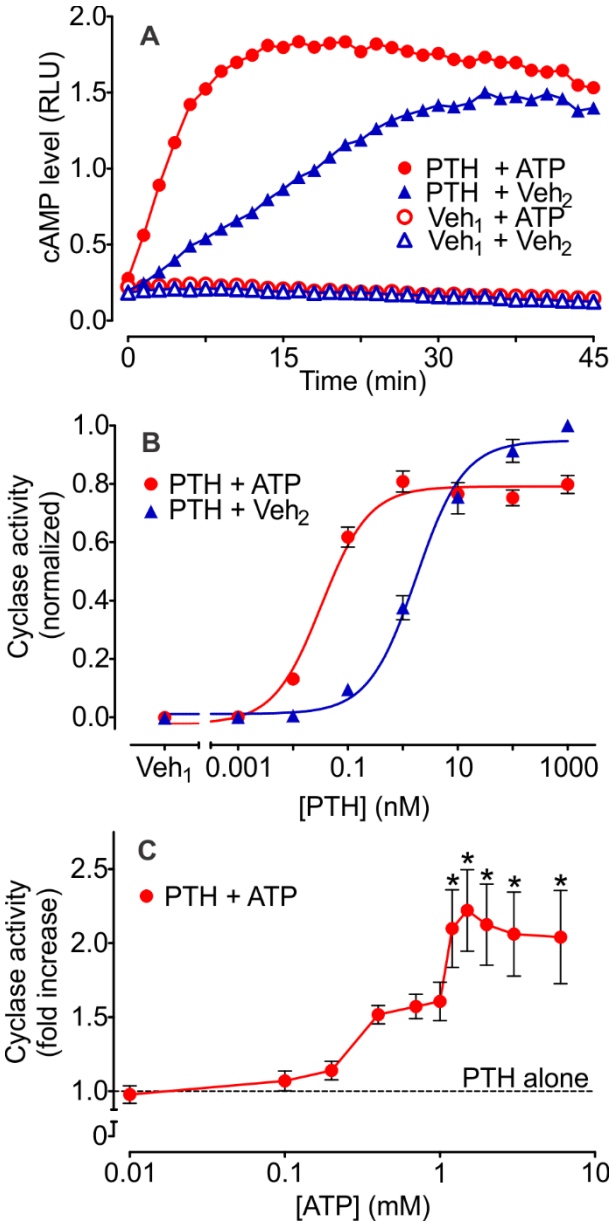
2.3 Results

2.3.1 Extracellular ATP enhances PTH-induced adenylyl cyclase activity

We first investigated the effect of extracellular ATP on PTH-induced activation of adenylyl cyclase. UMR-106 cells, a rat osteoblast-like cell line that endogenously expresses PTH1R (Partridge et al., 1983), were transfected with a plasmid encoding a luciferase-based cAMP biosensor. To suppress cAMP hydrolysis, cells were treated with the cAMP phosphodiesterase inhibitor IBMX. Cells were then stimulated with PTH (1-34) (1 nM) or its vehicle in the presence or absence of ATP (1.5 mM) (Fig. 2.1A). ATP alone did not produce a detectable change in cAMP levels. In contrast, PTH alone induced a steady increase in cAMP levels until reaching a plateau at approximately 30 min. Notably, ATP markedly enhanced the rate of PTH-induced cAMP accumulation.

We next evaluated how this effect of ATP depended on PTH concentration. In these experiments, cells were stimulated with various concentrations of PTH (1-34) in the presence or absence of 1.5 mM ATP. Adenylyl cyclase activity was determined from the time course data (*e.g.* Fig. 2.1A) as the maximal slope (Fig. 2.2). PTH alone yielded a sigmoidal concentration dependence curve with pEC_{50} of 8.75 ± 0.08 (mean ± SEM, Fig. 2.1B), consistent with previous reports (Thomas et al., 2009). In the presence of ATP, the concentration dependence curve was strikingly shifted to the left (pEC_{50}= 10.51 ± 0.09), corresponding to an approximately 60-fold increase in measured potency. Next, we evaluated how enhancement of PTH-induced adenylyl cyclase activity depended on ATP concentration. Potentiation of adenylyl cyclase activity was significant at ATP concentrations greater that 1 mM (Fig. 2.1C), clearly distinct from the concentration dependence previously reported for crosstalk between PTH1R and P2

Fig. 2.1. Extracellular ATP enhances PTH-induced activation of adenylyl cyclase: dependence on PTH and ATP concentrations. (**A**) UMR-106 osteoblastic cells were transfected with GloSensor™ cAMP biosensor plasmid. Cells were treated with IBMX. At time 0, cells were stimulated with PTH (1-34) (PTH, 1 nM) or its vehicle (Veh_1) in the presence of ATP (1.5 mM) or its vehicle (Veh_2). Luminescence intensity, which corresponds to the level of cytosolic cAMP, was measured from live cells every 1.5 min. Panel illustrates the results of an individual experiment, representative of 21 independent experiments. (**B**) Cells were stimulated with the indicated concentration of PTH (or its vehicle, Veh_1) in the presence of ATP (1.5 mM) or its vehicle (Veh_2). Adenylyl cyclase activity was determined as the maximal slope of each cAMP *vs* time curve (*e.g. panel A*) as described in Fig. 2.2. Data were normalized as a fraction of the greatest value of cyclase activity in each experiment. Values are means ± SEM (n = 3 independent experiments, each performed in duplicate). pEC_{50} for PTH alone was 8.75 ± 0.08 and for PTH in the presence of ATP was 10.51 ± 0.09; these two values differed significantly ($p < 0.0001$, based on extra sum-of-squares F-test). (**C**) Cells were stimulated with PTH (1 nM) in the presence of the indicated concentration of ATP or its vehicle (PTH alone). Data were normalized to adenylyl cyclase activity in the absence of ATP. Values are means ± SEM (n = 5 independent experiments). * indicates significant difference from PTH alone ($p < 0.05$, based on one-way ANOVA and Bonferroni test).


A
cAMP level (RLU)
2.0
1.5
1.0
0.5
0.0
0
15
30
45
Time (min)
PTH + ATP
PTH + Veh2
Veh1 + ATP
Veh1 + Veh2
B
Cyclase activity (normalized)
1.0
0.8
0.6
0.4
0.2
0.0
PTH + ATP
PTH + Veh2
Veh1
0.001
0.1
10
1000
[PTH] (nM)
C
Cyclase activity (fold increase)
2.5
2.0
1.5
1.0
0
PTH + ATP
PTH alone
0.01
0.1
1
10
[ATP] (mM)

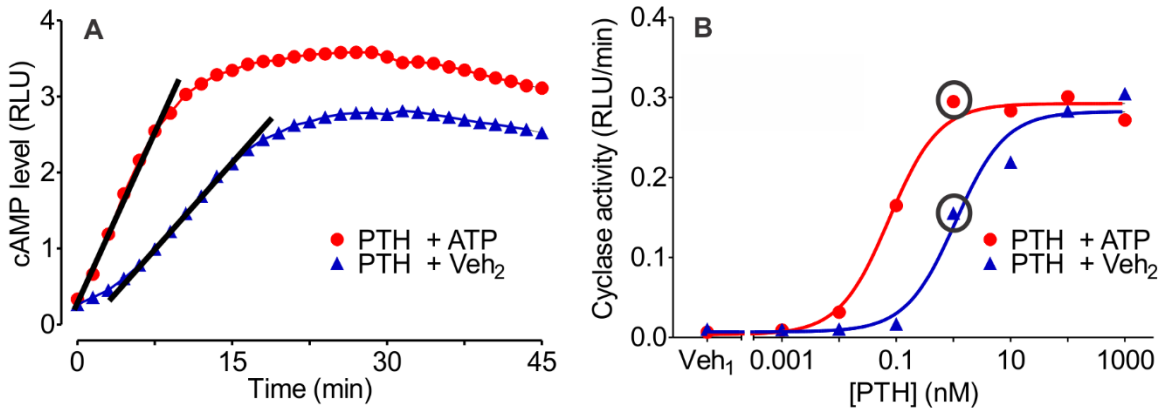


Fig. 2.2. Determination of maximal adenylyl cyclase activity from cAMP level vs time data. UMR-106 osteoblastic cells were transfected with GloSensor™ cAMP biosensor plasmid as described in *Materials and Methods*. Cells were treated with the cAMP phosphodiesterase inhibitor IBMX (500 µM). Data illustrate the results of an individual representative experiment. (**A**) At time 0, cells were stimulated with PTH (1-34) (PTH, 1 nM) in the presence of ATP (1.5 mM) or its vehicle (Veh_2). Luminescence intensity, which corresponds to the level of cytosolic cAMP, was measured from live cells every 1.5 min. PTH elevated the level of cAMP in a time-dependent manner (closed blue triangles). Extracellular ATP enhanced the rate of cAMP accumulation (closed red circles). Adenylyl cyclase activity was determined from each curve as the maximal slope. Maximal slopes are illustrated in this case by the *black lines*. (**B**) To investigate concentration dependence relationships, cells were stimulated with the indicated concentration of PTH (or its vehicle, Veh_1) in the presence of ATP (1.5 mM) or its vehicle (Veh_2). Adenylyl cyclase activity was determined as the maximal slope of each cAMP *vs* time curve (as described in panel **A**). Concentration dependence data were fitted to a 3-parameter sigmoidal equation using nonlinear regression. Data points corresponding to the slopes shown in panel **A** are indicated with *black circles*.

purinoceptors (Kaplan et al., 1995).

We confirmed the effect of ATP on PTH-induced adenylyl cyclase activity using an end-point cAMP immunoassay. Again, ATP induced a significant shift to the left in the PTH concentration dependence curve (Fig. 2.3), consistent with results from the kinetic biosensor assay shown in Fig. 2.1 and, thus, arguing against a potential artefact arising from ATP interference with the bioluminescence reaction.

To assess whether ATP-induced potentiation is specific to UMR-106 cells, we used the cAMP biosensor assay to investigate the effect of ATP on PTH1R signaling in other cell types. As in UMR-106 cells, ATP potentiated the effects of PTH (1-34) in the murine osteoblast-like cell line MC3T3-E1, which endogenously expresses PTH1R (Wang et al., 1999), and in HEK293H cells transfected with human PTH1R (Fig. 2.4).

2.3.2 ATP enhances PTH-induced cAMP accumulation in the absence of phosphodiesterase inhibitor

We next assessed whether extracellular ATP enhances PTH-induced cAMP accumulation under conditions that permitted both the synthesis and degradation of cAMP, thereby mimicking normal physiological conditions. We again used UMR-106 cells transfected with the cAMP biosensor plasmid but, in these experiments, cells were not treated with IBMX. At time 0, cells were stimulated with various concentrations of PTH (1-34) in the presence or absence of ATP. As expected, cAMP levels peaked and then declined (Fig. 2.5A, B). Under these conditions, ATP

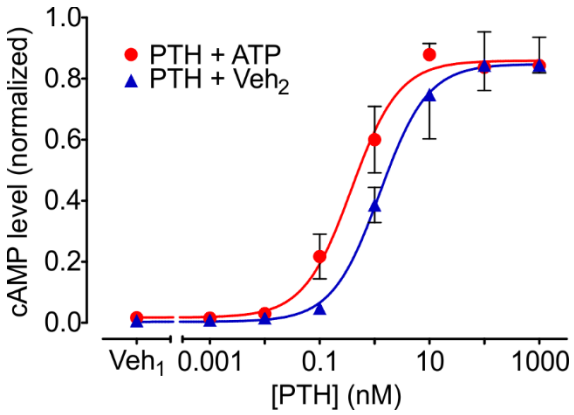


Fig. 2.3. End-point cAMP immunoassay confirms that ATP enhances PTH-induced cAMP accumulation. In this series of experiments, cAMP levels were measured using a cAMP-Screen™ Cyclic AMP Immunoassay System as described in *Materials and Methods*. UMR-106 osteoblastic cells were treated with the cAMP phosphodiesterase inhibitor IBMX (500 µM). Next, cells were stimulated with indicated concentration of PTH or vehicle (Veh_1) in the presence of ATP (1.5 mM) or its vehicle (Veh_2). After incubation for 10 min, cell lysates were collected and emitted luminescence was measured. Data shown correspond to cAMP levels under each condition and were normalized as a fraction of the greatest value in each experiment. Values are means ± SEM (n = 3 independent experiments, each performed in duplicate). The effect of ATP was significant ($p < 0.05$, based on extra sums of squares F-test). These data reveal that ATP enhances PTH-induced adenylyl cyclase activity, consistent with the results of the kinetic biosensor assay (Fig. 2.1).

Fig. 2.4. Extracellular ATP enhances PTH-induced elevation of cytosolic cAMP in MC3T3-E1 and HEK293H cells. (**A**) MC3T3-E1 osteoblastic cells were transfected with GloSensor™ cAMP biosensor plasmid as described under *Materials and Methods*. Cells were treated with two cAMP phosphodiesterase inhibitors Ro 20-1724 (200 μM) and IBMX (500 μM). Next, cells were stimulated with PTH (1-34) (PTH, 1 nM) or its vehicle (Veh_1) in the presence of ATP (1 mM) or its vehicle (Veh_2). Luminescence intensity, which corresponds to the level of cytosolic cAMP, was measured from live cells every 1.5 min for 45 min. There was no detectable response to vehicles alone (open blue triangles) or ATP alone (open red circles). In contrast, PTH elevated the level of cAMP in a time-dependent manner (closed blue triangles). Extracellular ATP enhanced the initial rate of cAMP accumulation (closed red circles). Graph illustrates the results of an individual experiment, representative of 3 independent experiments performed in triplicate. (**B**) HEK293H cells were co-transfected with two plasmids – pcDNA3.1(+)-hPTH1R, which encodes PTH1R, and the GloSensor™ cAMP biosensor plasmid – as described in *Materials and Methods*. Cells were treated with IBMX (500 μM) and stimulated with PTH (1-34) (PTH, 1 nM) or its vehicle (Veh_1) in the presence of ATP (1.5 mM) or its vehicle (Veh_2). Once again, PTH elevated the level of cAMP in a time-dependent manner (closed blue triangles) and ATP enhanced the rate of cAMP accumulation (closed red circles). Graph illustrates the results of an individual experiment, representative of 3 independent experiments, performed in duplicate. **(C)** HEK293H cells were stimulated with the indicated concentration of PTH (or its vehicle, Veh_1) in the presence of ATP (1.5 mM) or its vehicle (Veh_2). Data were normalized as a fraction of the greatest value of cyclase activity in each experiment. Values are means ± SEM (n = 3 independent experiments, each performed in duplicate). pEC_{50} for PTH alone was 8.93 ± 0.11 and for PTH + ATP was 10.25 ± 0.05, indicating that ATP induced a significant increase in apparent potency ($p < 0.0001$, based on extra sum-of-squares F-test).

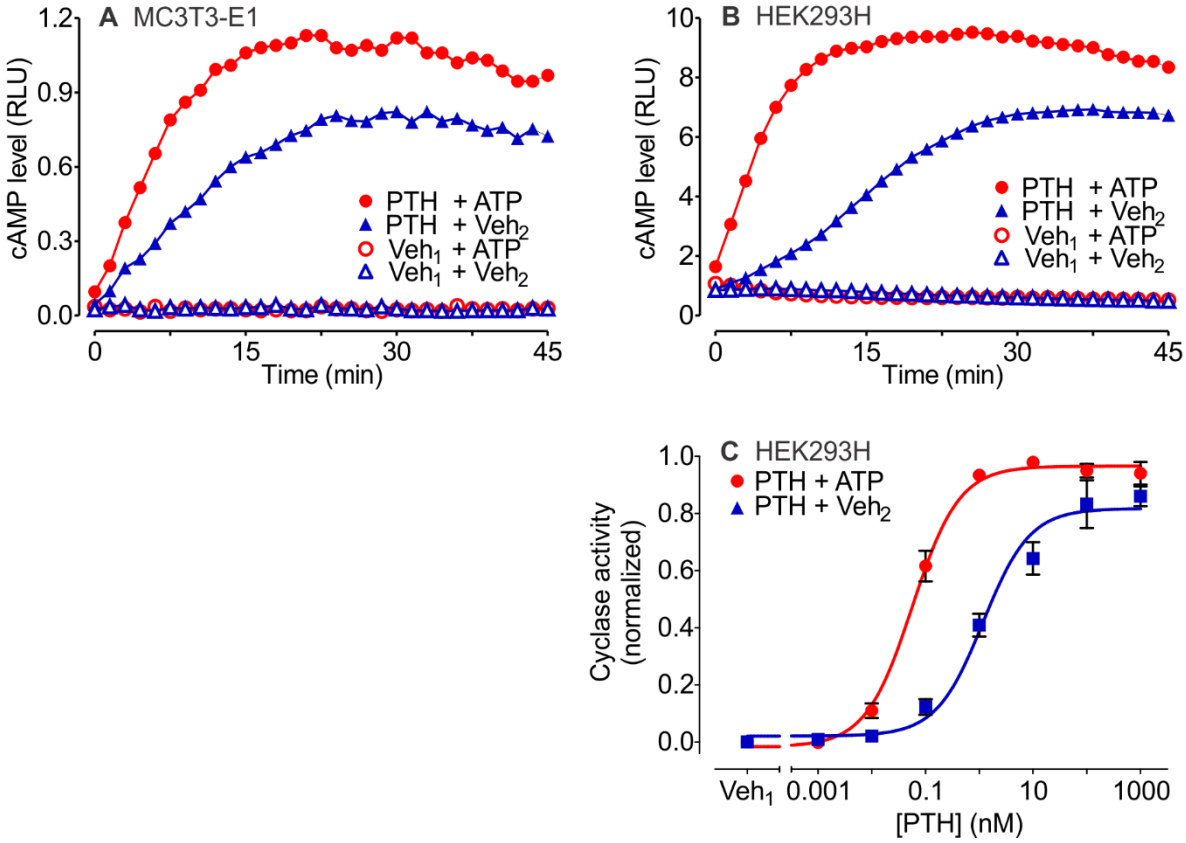
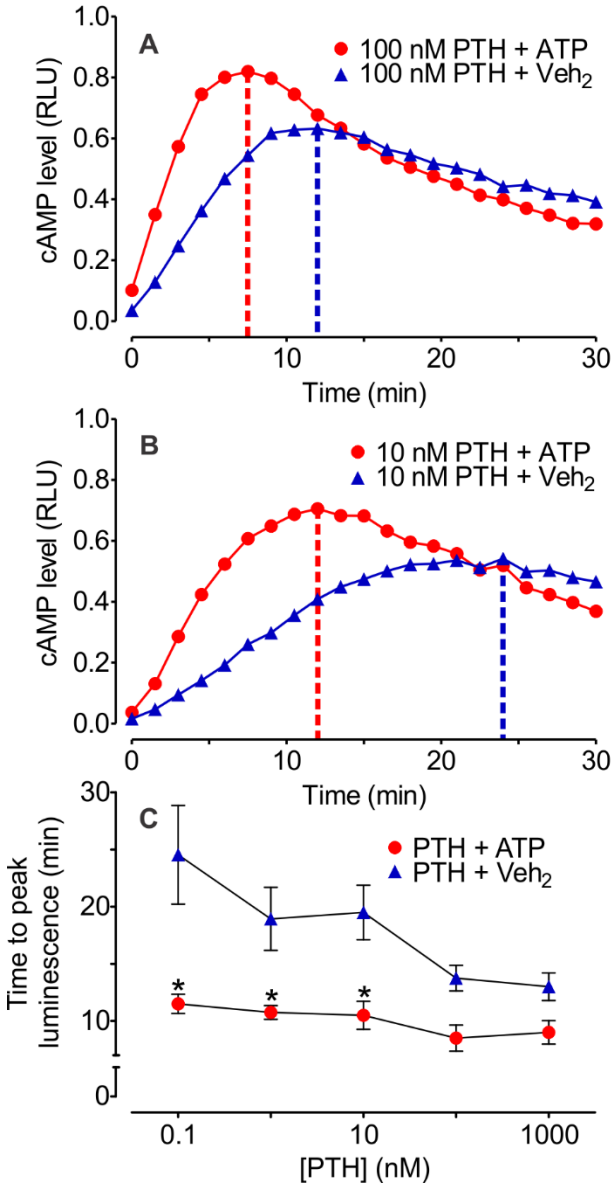

A MC3T3-E1
cAMP level (RLU)
1.2
0.9
0.6
0.3
0.0
0
15
30
45
Time (min)
PTH + ATP
PTH + Veh2
Veh1 + ATP
Veh1 + Veh2
B HEK293H
cAMP level (RLU)
10
8
6
4
2
0
0
15
30
45
Time (min)
PTH + ATP
PTH + Veh2
Veh1 + ATP
Veh1 + Veh2
C HEK293H
PTH + ATP
PTH + Veh2
Cyclase activity (normalized)
1.0
0.8
0.6
0.4
0.2
0.0
Veh1
0.001
0.1
10
1000
[PTH] (nM)

Fig. 2.5. In the absence of phosphodiesterase inhibitor, ATP potentiates and alters the kinetics of PTH-induced cAMP accumulation. UMR-106 cells were transfected with GloSensor™ cAMP biosensor plasmid. At time 0, cells were stimulated with the indicated concentrations of PTH in the presence of ATP (1.5 mM) or its vehicle (Veh_2). (**A** and **B**) PTH alone elevated cAMP levels, which peaked and then declined. ATP markedly enhanced PTH-induced cAMP accumulation. Moreover, ATP shifted time-to-peak to the left (*cf.* vertical red and blue dashed lines indicating time-to-peak in the presence and absence of ATP, respectively). Data are representative of at least 3 independent experiments, each performed in duplicate. (**C**) Time-to-peak was evaluated for each concentration of PTH in the presence and absence of ATP. Data are means ± SEM (n = 6 samples from 3 independent experiments). Overall, ATP had a marked effect on time-to-peak ($p < 0.001$, based on two-way ANOVA). * indicates significant effect of ATP at the same concentration of PTH ($p < 0.05$, based on Bonferroni test).


A
100 nM PTH + ATP
100 nM PTH + Veh2
cAMP level (RLU)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (min)
B
10 nM PTH + ATP
10 nM PTH + Veh2
cAMP level (RLU)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (min)
C
PTH + ATP
PTH + Veh2
Time to peak luminescence (min)
30
20
10
0
*
*
*
0.1
10
1000
[PTH] (nM)

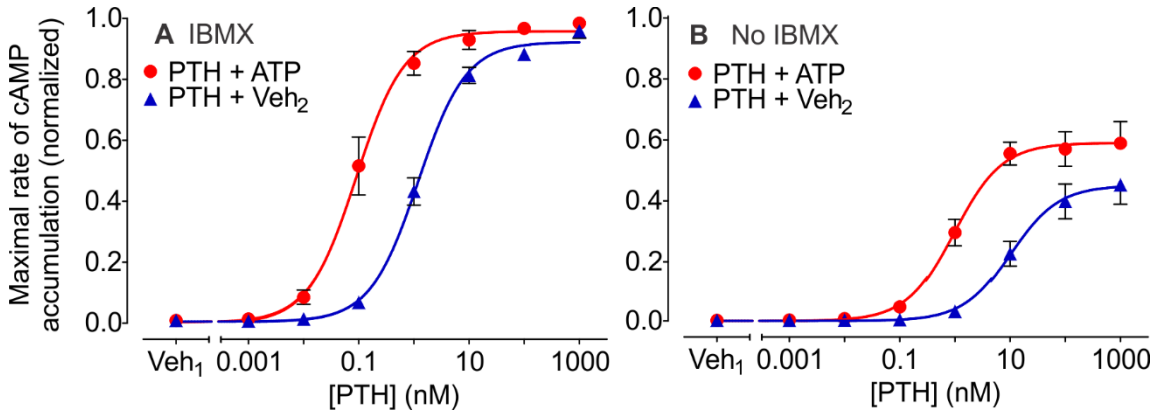


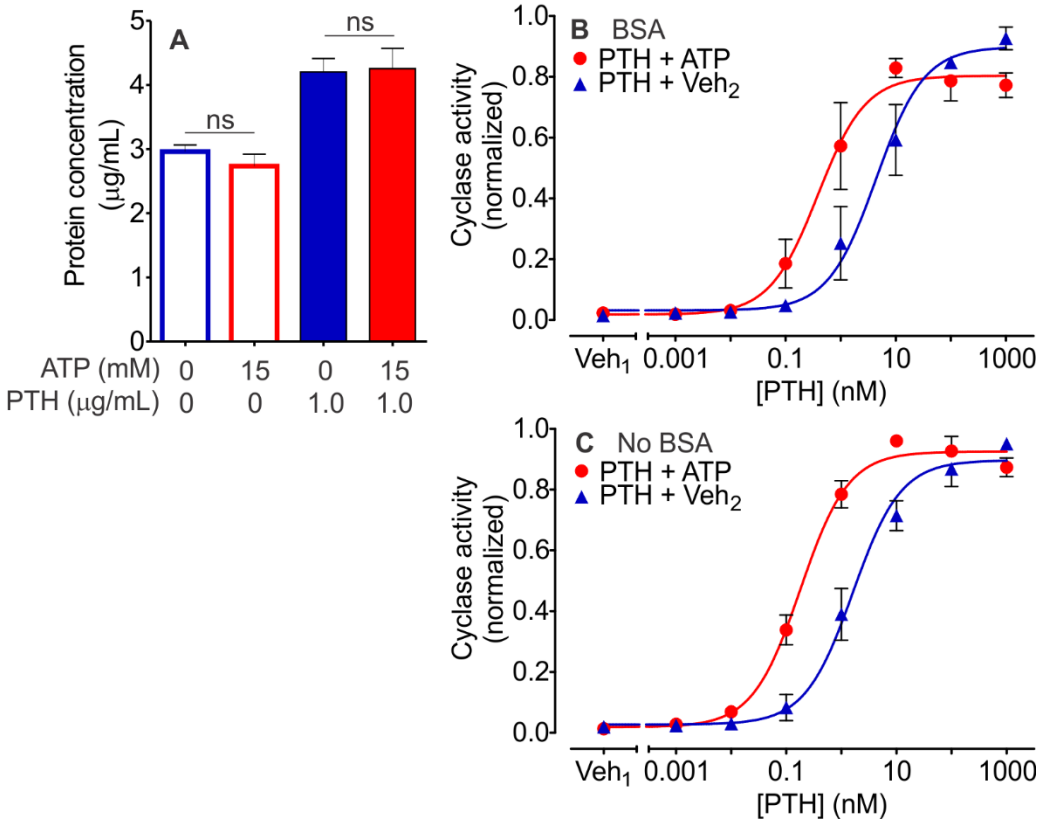
Fig. 2.6. ATP enhances PTH-induced elevation of cAMP in both the presence and absence of phosphodiesterase inhibitor IBMX. UMR-106 osteoblastic cells were transfected with GloSensor™ cAMP biosensor plasmid as described under *Materials and Methods*. In each experiment, parallel samples of cells were treated with cAMP phosphodiesterase inhibitor IBMX (500 µM) (**A**) or its vehicle (**B**). Next, cells were stimulated with the indicated concentration of PTH (or its vehicle, Veh_1) in the presence of ATP (1.5 mM) or its vehicle (Veh_2). Data were normalized as a fraction of the maximal rate of cAMP accumulation in each experiment (note that data in panel *B* are normalized as a fraction of the maximum in panel **A**). Values are means ± SEM (n = 3 independent experiments, data illustrated in Fig. 2.5 are from these same three experiments). ATP induced a significant increase in apparent potency, in both the presence and absence of IBMX. In the presence of IBMX, pEC_{50} for PTH alone was 8.93 ± 0.05 and for PTH + ATP was 10.05 ± 0.08. In the absence of IBMX, pEC_{50} for PTH alone was 7.99 ± 0.13 and for PTH + ATP was 9.01 ± 0.11. In both cases, ATP induced a significant increase in apparent potency ($p < 0.0001$, based on extra sums of squares F-test).

still markedly potentiated cAMP accumulation. When the maximal rate of cAMP accumulation was evaluated at different PTH concentrations, we again observed that ATP induced a significant shift to the left in the PTH concentration dependence curve (Fig. 2.6).

Interestingly, cAMP accumulation reached maximal levels earlier in the presence of ATP than in its absence (see *vertical dashed lines* in Fig. 2.5A, B). This difference was statistically significant over the range of PTH concentrations from 0.1 to 10 nM (Fig. 2.5C). Although the underlying mechanism is unclear, these data suggest that ATP fundamentally alters the kinetics of PTH1R-induced cAMP accumulation. It is conceivable that the effect of ATP presented in Fig. 2.1 could be explained by ATP increasing the availability of free PTH; however, the data presented in Fig. 2.5 establish that ATP alters signaling kinetics in a manner that cannot be explained simply by an increase in the availability of free PTH.

We performed additional experiments to investigate whether the effects of ATP could be mediated indirectly by changes in the concentration of PTH available for receptor activation. Generally, experiments were performed in the presence of bovine albumin (BSA), which is added to buffers to improve the stability of peptides such as PTH and minimize nonspecific binding to plasticware (Goebel-Stengel et al., 2011). First, ATP had no observable effect on the proportion of protein bound to plasticware (Fig. 2.7A). In addition, ATP potentiated adenylyl cyclase activity in both the presence and absence of BSA (Fig. 2.7B, C). Taken together, these findings indicate that potentiation cannot simply be due to ATP altering the binding of PTH to either BSA or the plasticware used in experiments.

Fig. 2.7. ATP enhances PTH-induced elevation of cytosolic cAMP in both the presence and absence of extracellular BSA. We used two approaches to investigate whether the effects of ATP could be mediated by changing the concentration of PTH available for receptor activation. (**A**) First, we examined whether ATP could change the availability of PTH by altering the proportion of PTH in the soluble phase *versus* that bound to plastic. PTH (1 μg/mL) or its vehicle was mixed with ATP (15 mM) or its vehicle (all conditions in the presence of 2.5 μg/mL BSA). A concentration of 15 mM ATP was used in these experiments to mimic the concentration used when nucleotide was initially mixed with PTH for the live cell cAMP and β-arrestin-1-PTH1R interaction assays. Following incubation of samples, an aliquot was removed and protein content (representing protein in the soluble phase) was measured using Bradford protein assay. Values are means ± SEM (n = 3 independent experiments, each with 2-4 replicates). There was no significant effect of ATP on protein concentration (p = 0.7, based on two-way ANOVA), indicating that ATP does not markedly alter the proportion of BSA or PTH bound to plastic. (**B** and **C**) Second, we examined whether the ability of ATP to potentiate cyclase activity was dependent on the presence of BSA. UMR-106 osteoblastic cells were transfected with GloSensor™ cAMP biosensor plasmid as described under *Materials and Methods*. After 24 hour incubation at 37°C/5% CO_2, cells were incubated in MEM supplemented with D-luciferin and HEPES, but not BSA, and were incubated for two hours at room temperature. IBMX (500 μM) was then added and cells were further incubated for 20 min at room temperature. In each experiment, parallel samples of cells were stimulated (time 0) with PTH (nominal concentrations indicated), dissolved in either 0.1% BSA (**B**) or buffer without BSA (**C**). Concomitantly, samples were treated with ATP (1.5 mM) or its vehicle (Veh_2). Data were normalized as a fraction of the greatest value of cyclase activity in each experiment. Values are means ± SEM (n = 3 independent experiments). ATP induced a significant increase in apparent potency, in both the presence and absence of BSA ($p < 0.0001$, based on extra sums of squares F-test). In the presence of BSA, pEC_{50} for PTH alone was 8.36 ± 0.14 and for PTH + ATP was 9.42 ± 0.15. In the absence of BSA, pEC_{50} for PTH alone was 8.79 ± 0.10 and for PTH + ATP was 9.74 ± 0.07.


A
Protein concentration (μg/mL)
ns
ns
ATP (mM) 0 15 0 15
PTH (μg/mL) 0 0 1.0 1.0
B BSA
PTH + ATP
PTH + Veh2
Cyclase activity (normalized)
Veh1 0.001 0.1 10 1000
[PTH] (nM)
C No BSA
PTH + ATP
PTH + Veh2
Cyclase activity (normalized)
Veh1 0.001 0.1 10 1000
[PTH] (nM)

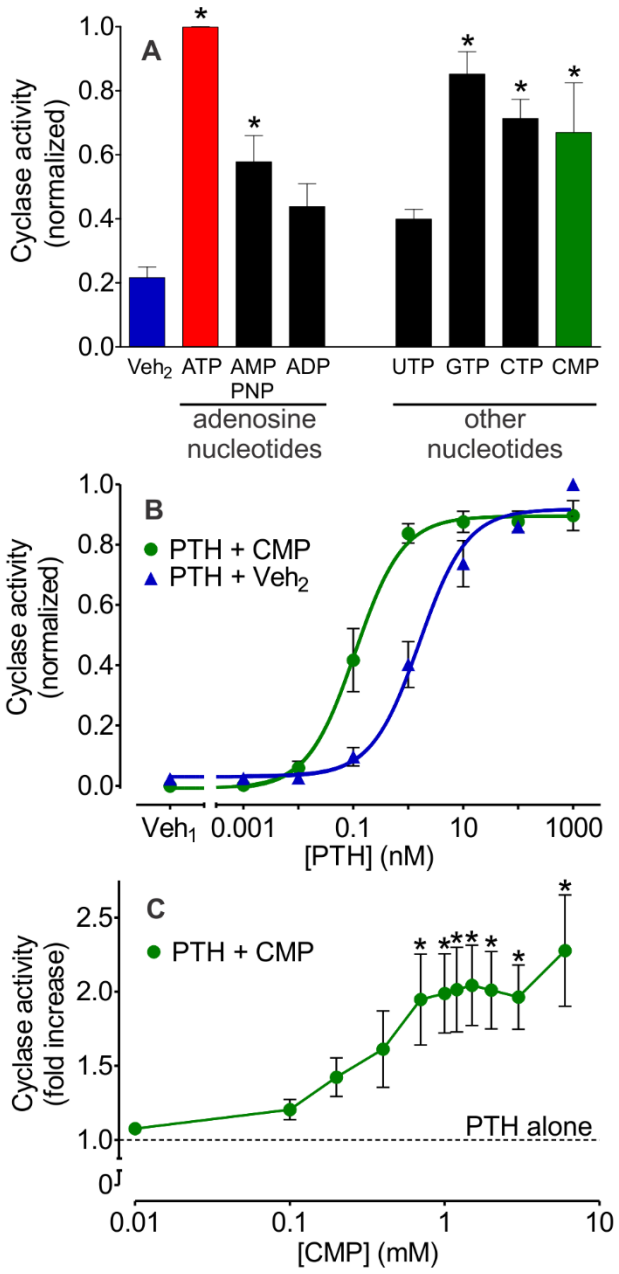
2.3.3 Nucleotide specificity for potentiation of PTH-induced adenylyl cyclase activity

To better understand the mechanism by which ATP facilitates PTH signaling and to begin to define the pharmacophore, we evaluated the activity of a number of nucleotides and analogs (Fig. 2.8A). ATP can serve as a substrate for ectokinases or ectonucleotidases. However, both CMP and the sparingly hydrolysable ATP analog AMP-PNP potentiated PTH-stimulated cAMP production in UMR-106 cells, arguing against a mechanism involving hydrolysis of a high-energy phosphoanhydride bond.

In addition, extracellular ATP and certain other nucleotides such as ADP and UTP serve as agonists for various cell-surface P2 receptors. However, several pieces of evidence rule out the involvement of P2 receptors. First, ADP and UTP failed to induce potentiation (Fig. 2.8A). Second, significant potentiation was observed in response to GTP, CTP and CMP, none of which are generally recognized as P2 receptor agonists[*]. Third, the relatively nonspecific P2 receptor antagonists, suramin and PPADS (Ralevic and Burnstock, 1998), failed to block potentiation (Fig. 2.9). Fourth, the observed concentration dependence for ATP (Fig. 2.1B) is inconsistent with most P2 receptors, with the exception of the low affinity P2X7 receptor. However, we ruled out involvement of P2X7 based on the lack of effect of A438079 (Fig. 2.9), a selective P2X7 antagonist (Donnelly-Roberts and Jarvis, 2007), and the presence of potentiation in cells lacking P2X7 (HEK293H and UMR-106 cells).

[*] In UMR-106 cells, we confirmed that CMP (1.5 mM, on its own) failed to induce an increase in either cytosolic Ca^{2+} (Fig. 2.10) or cAMP (Fig. 2.8B), arguing against activation of P2 receptors under these conditions.

Fig. 2.8. Effects of nucleotides on PTH-induced activation of adenylyl cyclase. UMR-106 cells were transfected with GloSensor™ cAMP biosensor plasmid and treated with IBMX. (**A**) Adenylyl cyclase activity was determined in parallel for PTH (0.1 nM) in the presence of the indicated nucleotide or analog (1.5 mM) or vehicle (Veh_2). The poorly hydrolysable ATP analog adenosine 5′-(β,γ-imido)triphosphate is abbreviated AMP-PNP. Data were normalized as a fraction of the greatest value of cyclase activity in each experiment. In the absence of PTH, none of the nucleotides tested altered adenylyl cyclase activity. Values are means ± SEM (n = 3 independent experiments, each performed in triplicate). * indicates significant difference from PTH alone ($p < 0.05$, based on one-way ANOVA and Bonferroni test). (**B**) Cells were stimulated with the indicated concentrations of PTH (or its vehicle, Veh_1) in the presence of CMP (1.5 mM) or its vehicle (Veh_2). Values are means ± SEM (n = 4 independent experiments, each performed in duplicate). pEC_{50} for PTH alone was 8.80 ± 0.10, and for PTH in the presence of CMP was 9.97 ± 0.10, a significant increase in apparent potency in the presence of CMP ($p < 0.0001$, based on extra sum-of-squares F-test). (**C**) Cells were stimulated with PTH (1 nM) in the presence of the indicated concentration of CMP or its vehicle (PTH alone). Data were normalized to adenylyl cyclase activity in the absence of CMP and are expressed as fold increases. Values are means ± SEM (n = 5 independent experiments, each performed in triplicate). * indicates significant difference from PTH alone ($p < 0.05$, based on one-way ANOVA and Bonferroni test).


A
Cyclase activity (normalized)
1.0
0.8
0.6
0.4
0.2
0.0
Veh2
ATP
AMP PNP
ADP
UTP
GTP
CTP
CMP
adenosine nucleotides
other nucleotides
B
PTH + CMP
PTH + Veh2
Cyclase activity (normalized)
Veh1
0.001
0.1
10
1000
[PTH] (nM)
C
PTH + CMP
Cyclase activity (fold increase)
2.5
2.0
1.5
1.0
0
PTH alone
0.01
0.1
1
10
[CMP] (mM)

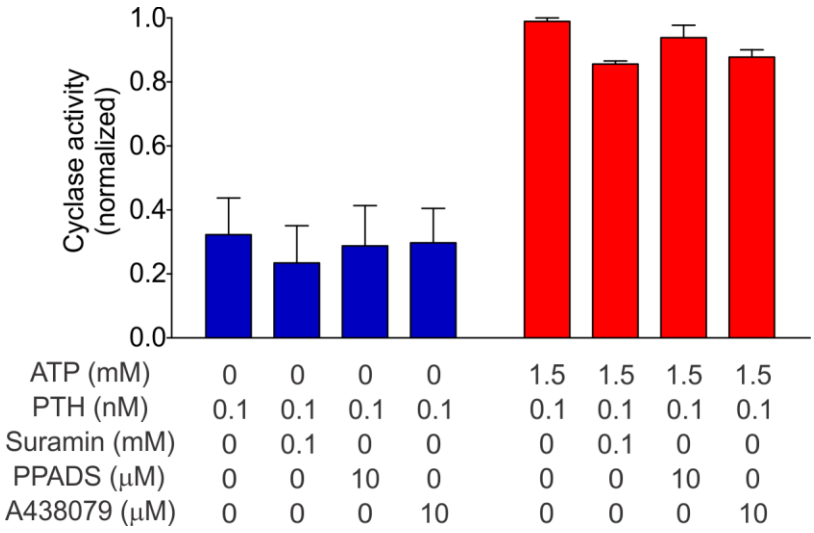


Fig. 2.9. Lack of effect of P2 receptor antagonists on ATP-induced potentiation. UMR-106 cells were transfected with GloSensor™ cAMP biosensor as described in *Materials and Methods*. Cells were treated with IBMX (500 μM). In each experiment, parallel samples of cells were treated for 20 min with a non-selective P2 receptor antagonist suramin (0.1 mM) or PPADS (10 μM), the selective P2X7 inhibitor A438079 (10 μM), or vehicle. Next, cells were stimulated with PTH (1-34, 0.1 nM) in the presence or absence of ATP (1.5 mM). Data were normalized as a fraction of the greatest value of cyclase activity in each experiment. Values are means ± SEM (n = 3 independent experiments, each performed in triplicate). In each case, ATP significantly potentiated PTH-induced activation of adenylyl cyclase (*cf.* red bars to blue bars, $p < 0.001$); in contrast, P2 receptor antagonists had no significant effects ($p = 0.62$, based on two-way ANOVA). These and other data argue that ATP enhances PTH-induced adenylyl cyclase activity independently of P2 nucleotide receptors.

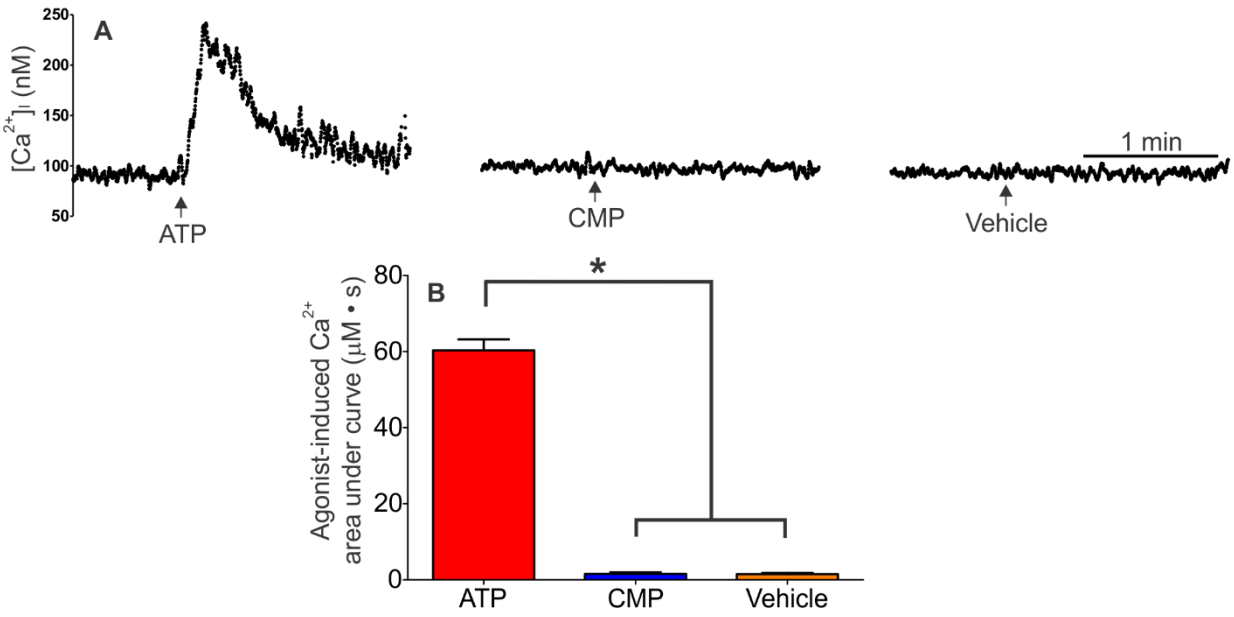


Fig. 2.10. CMP does not elevate cytosolic Ca2+ concentration in UMR-106 cells. UMR-106 cells were loaded with Ca^{2+}-sensitive probe indo-1 and changes in $[Ca^{2+}]_i$ were monitored as described in *Materials and Methods.* (**A**) Where indicated, cells were stimulated with ATP, CMP (1.5 mM) or Vehicle. Representative traces demonstrate that ATP induced transient elevation in $[Ca^{2+}]_i$, which was not observed in response to CMP or vehicle. Addition artefacts were removed from traces. (**B**) Calcium responses were quantified by calculating the area under the curve. Data are means ± SEM ($n \geq 50$ samples from at least 12 separate experiments). * indicates significant effect of ATP ($p < 0.05$). There was no significant difference between the responses to CMP and vehicle.

For more detailed study, we chose CMP, a nucleotide which potentiates PTH signaling, but lacks a high energy phosphate and the ability to activate P2 receptors. Like ATP, CMP increased the apparent potency of PTH (Fig. 2.8B). Moreover, CMP induced significant potentiation at concentrations of 0.8 mM and greater (Fig. 2.8C), similar to the concentration dependence of ATP. Taken together, these data rule out the possible involvement of ectokinases or P2 receptors in mediating potentiation. Rather, the mechanism appears to involve an allosteric interaction with PTH, its receptor, or an accessory protein.

2.3.4 Nucleotides enhance PTH-induced recruitment of β-arrestin-1 to PTH1R

We next examined the effect of ATP on PTH-induced recruitment of β-arrestin to PTH1R, which is known to be independent of G proteins (Cheloha et al., 2015). We monitored recruitment of β-arrestin-1 in real-time using a luciferase complementation assay (Misawa et al., 2010). HEK293H cells were transfected with plasmids encoding the biosensor and stimulated with PTH (1-34, 1 nM) in the presence or absence of ATP or CMP (1.5 mM). Nucleotides alone had little effect, however, they markedly enhanced PTH-induced PTH1R-β-arrestin-1 coupling (Fig. 2.11A, B).

We next evaluated the dependence of β-arrestin-1 recruitment on the concentration of PTH. Maximal rates of recruitment were determined from the time course data. Both ATP and CMP increased the measured potency of PTH (1-34) to induce PTH1R-β-arrestin-1 coupling (Fig. 2.11C), similar to their effects on adenylyl cyclase activity (Figs. 2.1B and 2.8B)[†]. Moreover,

[†] PTH (1-34) exhibited a markedly greater potency at PTH1R for activation of cyclase than for recruitment of β-arrestin, as reported for agonists at some other GPCRs Rajagopal, S., S. Ahn,

ATP and CMP significantly potentiated recruitment at concentrations of 1 mM and greater (Fig. 2.11D), similar to their concentration dependence for potentiation of adenylyl cyclase activity (Figs. 2.1C and 2.8C). Thus, it appears that nucleotides exert their effects on PTH1R signaling by binding to a target upstream of G proteins, arguing against possibilities such as direct interaction with the extracellular domain of adenylyl cyclase.

2.3.5 ATP increases the potency of other PTH1R agonists in addition to PTH (1-34)

We next tested whether the effects of ATP might extend to other PTH1R agonists. PTHrP is the product of a gene distinct from that encoding PTH that activates PTH1R. The sequence of PTHrP is not related closely to that of PTH except the first thirteen residues, of which eight are identical (Wysolmerski, 2012). Nevertheless, ATP potentiated the ability of PTHrP to activate adenylyl cyclase, shifting the concentration-dependence curve to the left (Fig. 2.12A). Next, we stimulated cells with PTH (1-14), a fragment PTH of that activates PTH1R with relatively low affinity (Luck et al., 1999). Again, ATP was found to potentiate agonist-dependent activation of adenylyl cyclase (Fig. 2.12B). Thus, ATP enhances the potency of multiple PTH1R agonists, arguing against the possibility of a direct interaction between ATP and PTH. Thus, it is likely that ATP acts directly on the receptor itself or a closely associated protein to modulate signaling.

In this regard, we considered the possibility that ATP might exert its effects through interaction with receptor activity modifying proteins (RAMPs) – single transmembrane spanning

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Fig. 2.11. Extracellular ATP and CMP enhance PTH-induced recruitment of β-arrestin-1 to PTH1R. (**A** and **B**) HEK293H cells were co-transfected with plasmids encoding N-terminal luciferase fragment-β-arrestin-1 and PTH1R-C-terminal luciferase fragment. Cells were stimulated with PTH (1 nM) or vehicle (Veh_1) in the presence of ATP, CMP (1.5 mM), or vehicle (Veh_2) as indicated. Luminescence intensity, which corresponds to β-arrestin-1 recruitment to PTH1R, was then measured from live cells every 2 min. Panels *A* and *B* illustrate the results of individual experiments, each representative of 3 independent experiments performed in duplicate. (**C**) Cells were stimulated with the indicated concentrations of PTH or with vehicle (Veh_1) in the presence of ATP, CMP (1.5 mM), or vehicle (Veh_2). The maximal rate of β-arrestin-1 recruitment was determined from the β-arrestin-1 recruitment *versus* time curves (*e.g*. panels **A** and **B**) as the greatest slope. Data were normalized as a fraction of the greatest rate of recruitment in each experiment. Values are means ± SEM (n = 3 independent experiments, each performed in duplicate). pEC_{50} for PTH alone was 8.11 ± 0.10. Both ATP and CMP significantly increased pEC_{50} (8.94 ± 0.07 for PTH + ATP and 8.84 ± 0.06 for PTH + CMP, $p < 0.0001$, based on extra sum-of-squares F-tests). (**D**) Cells were stimulated with PTH (10 nM) in the presence of the indicated concentrations of ATP, CMP, or their vehicle (PTH alone). Data were normalized to β-arrestin-1 recruitment in the absence of nucleotide and expressed as fold increases. Values are means ± SEM (n = 3 independent experiments each performed in duplicate). * indicates significant difference from PTH alone ($p < 0.05$, based on two-way ANOVA and Bonferroni test).

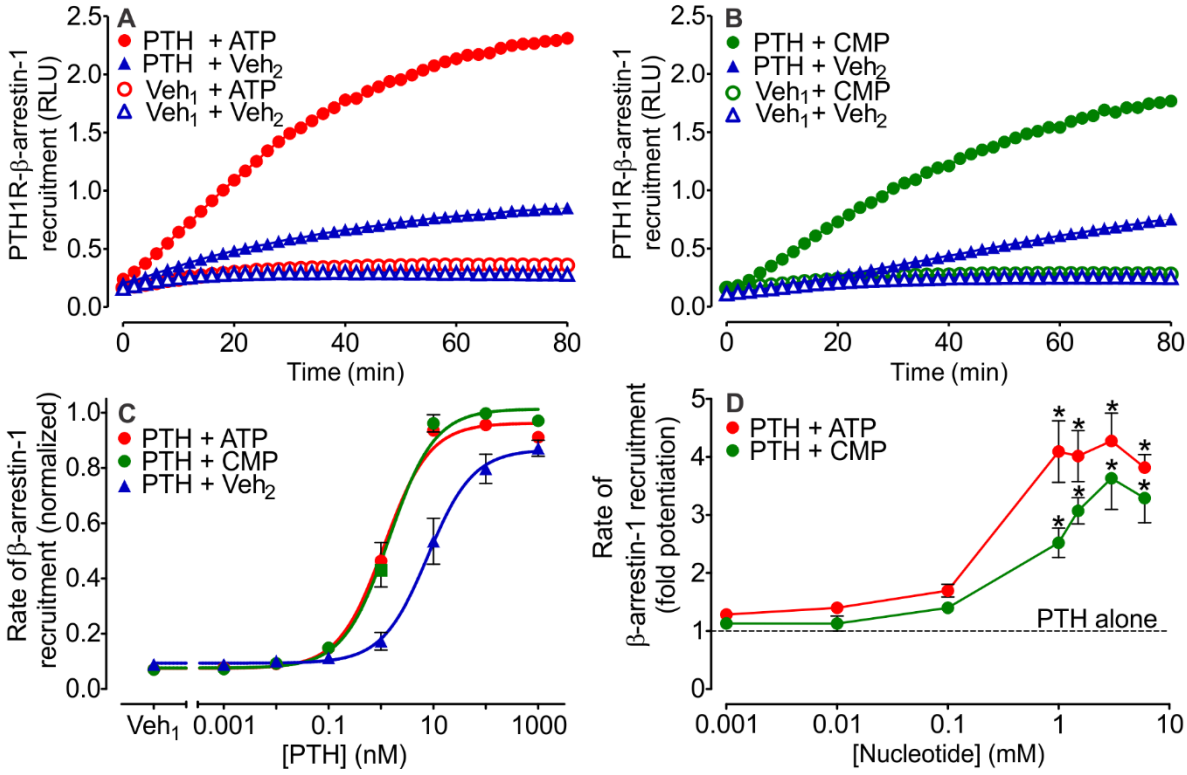
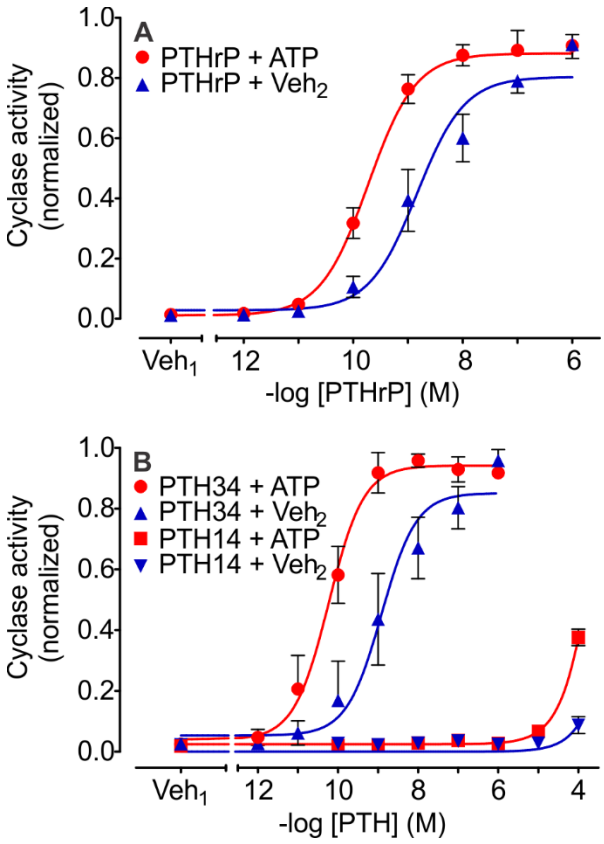

A
PTH + ATP
PTH + Veh2
Veh1 + ATP
Veh1 + Veh2
PTH1R-β-arrestin-1 recruitment (RLU)
Time (min)
B
PTH + CMP
PTH + Veh2
Veh1+ CMP
Veh1+ Veh2
PTH1R-β-arrestin-1 recruitment (RLU)
Time (min)
C
PTH + ATP
PTH + CMP
PTH + Veh2
Rate of β-arrestin-1 recruitment (normalized)
Veh1
[PTH] (nM)
D
PTH + ATP
PTH + CMP
Rate of β-arrestin-1 recruitment (fold potentiation)
PTH alone
[Nucleotide] (mM)

Fig. 2.12. ATP enhances activation of adenylyl cyclase induced by PTHrP and PTH (1-14). UMR-106 cells were transfected with GloSensor™ cAMP biosensor plasmid and treated with IBMX. (**A**) Cells were stimulated with indicated concentrations PTH-related peptide, PTHrP (1-34) or vehicle (Veh_1) in the presence of ATP (1.5 mM) or its vehicle (Veh_2). Note that the scale of the x-axis differs between the upper and lower panels. Values are means ± SEM (n = 3 independent experiments, each performed in duplicate). ATP significantly enhanced the potency of PTHrP (pEC_{50} shifted from 8.97 ± 0.14 to 9.82 ± 0.12) ($p < 0.0001$ based on extra sum-of-squares F-test). (**B**) Adenylyl cyclase activity was determined in parallel for the indicated concentrations of PTH (1-34) (PTH34) and the low affinity PTH1R agonist PTH (1-14) (PTH14) in the presence and absence of ATP (1.5 mM). Values are means ± SEM (n = 3 independent experiments, each performed in duplicate). ATP significantly enhanced the potency of PTH (1-34) (pEC_{50} shifted from 8.92 ± 0.20 to 10.23 ± 0.12, $p < 0.001$ based on extra sum-of-squares F-test). Moreover, ATP significantly enhanced the response to PTH (1-14), which at 100 µM increased cyclase activity from 0.09 ± 0.03 to 0.38 ± 0.03 ($p < 0.01$ based on two-tailed t-test).


A
PTHrP + ATP
PTHrP + Veh2
Cyclase activity (normalized)
1.0
0.8
0.6
0.4
0.2
0.0
Veh1
12
10
8
6
-log [PTHrP] (M)
B
PTH34 + ATP
PTH34 + Veh2
PTH14 + ATP
PTH14 + Veh2
Cyclase activity (normalized)
1.0
0.8
0.6
0.4
0.2
0.0
Veh1
12
10
8
6
4
-log [PTH] (M)

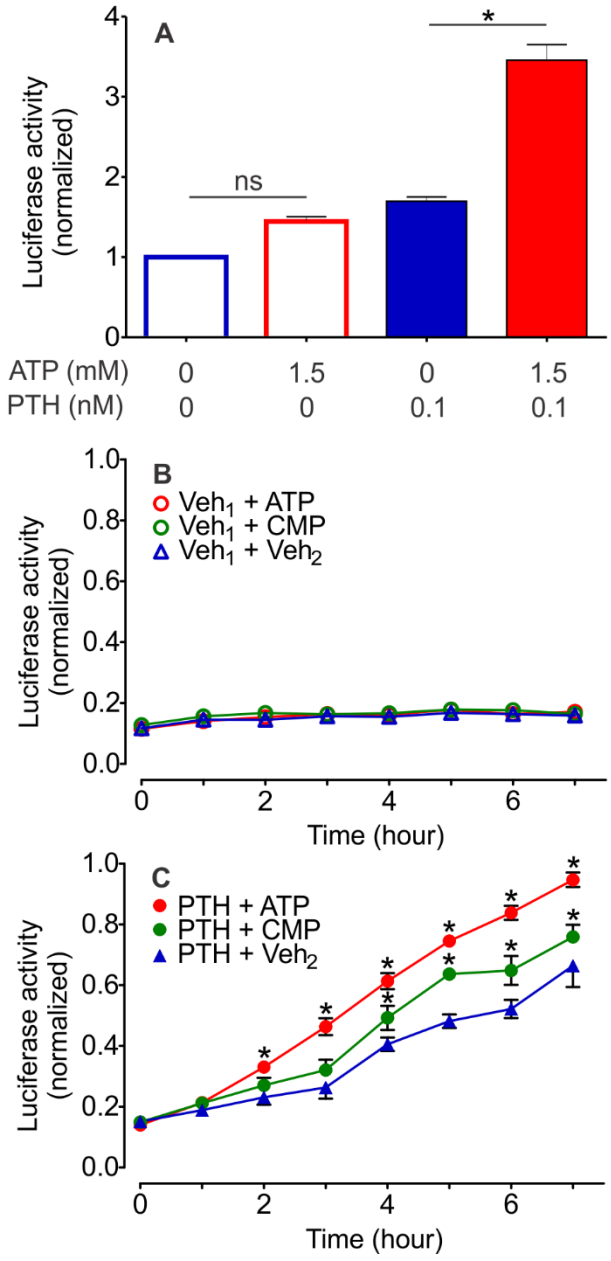
proteins that can alter agonist binding properties and trafficking GPCRs (Hay and Pioszak, 2016), including PTH1R (Christopoulos et al., 2003). To investigate the role of RAMP proteins, COS7 cells, which lack endogenous RAMP proteins (Bouschet et al., 2005), were transfected with RAMP and the effect of ATP on PTH1R signaling was assessed (Experiments were performed by Dr. Shuying Zhu). However, ATP potentiated the cAMP response to PTH similarly in both the presence and absence of RAMPs (RAMP1, RAMP2 or RAMP3), indicating that RAMPs are not essential for the effects of ATP on PTH signaling.[‡]

2.3.6 Nucleotides enhance the activation of CREB induced by PTH

We next investigated whether potentiation of PTH signaling by extracellular nucleotides might influence cell function. It is known that cAMP signaling promotes translocation of the catalytic subunit of PKA to the nucleus to activate CREB, leading to transcriptional regulation (Mayr and Montminy, 2001). UMR-106 cells were transfected with a CRE luciferase reporter. These experiments were performed in the absence of IBMX. Neither ATP nor CMP alone triggered significant CREB activity (Fig. 2.13A, B). As expected, PTH (1-34, 0.1 nM) alone activated CREB (Fig. 2.13A, C). Notably, activation of CREB by PTH was enhanced significantly by both ATP and CMP. Thus, the potentiating effect of nucleotides on upstream signaling events is reflected in downstream events such as the activation of transcription factors.

[‡] As nucleotides can chelate Mg^{2+}, we also investigated the possible involvement of extracellular Mg^{2+} in mediating ATP-induced potentiation. A similar degree of potentiation was observed in the presence and absence of extracellular Mg^{2+}, arguing against its involvement.

Fig. 2.13. ATP and CMP enhance PTH-induced CREB activation. UMR-106 cells were transfected with a CRE luciferase reporter plasmid as described under *Materials and Methods*. (**A**) Cells were incubated for 24 h in serum containing medium. In the absence of IBMX, cells were then stimulated with PTH (1-34) (0.1 nM) or its vehicle (Veh_1) in the presence of ATP (1.5 mM) or vehicle (Veh_2). After an additional 24 h incubation, cell lysates were collected and luminescence was measured. Data were normalized to luciferase activity in the absence of ATP and PTH, and expressed as fold increases. Values are means ± SEM (n = 3 independent experiments, each performed in duplicate). * indicates a significant effect of ATP ($p < 0.05$, based on one-way ANOVA and Bonferroni test). (**B**, **C**) Parallel samples of cells were incubated in serum containing medium for the first 24 h, and in serum-free medium for the final 24 h. In the absence of IBMX, cells were then stimulated with vehicle (Veh_1, **B**) or PTH (0.1 nM, **C**) in the presence of ATP, CMP (1.5 mM) or vehicle (Veh_2). At the times indicated, cell lysates were collected and luminescence was measured. Data were normalized as a fraction of the greatest luciferase activity in each experiment. Values are means ± SEM (n = 6 or 8 samples from 3 or 4 independent experiments, respectively). *indicates significant difference compared to PTH + veh_2 ($p < 0.05$, based on two-way ANOVA and Bonferroni test).


A
Luciferase activity (normalized)
ns
*
ATP (mM) 0 1.5 0 1.5
PTH (nM) 0 0 0.1 0.1
B
Veh1 + ATP
Veh1 + CMP
Veh1 + Veh2
Luciferase activity (normalized)
Time (hour)
C
PTH + ATP
PTH + CMP
PTH + Veh2
Luciferase activity (normalized)
Time (hour)

2.4 Discussion

ATP is well known as an allosteric modulator. For example, the activities of many cellular enzymes are increased or decreased according to intracellular ATP levels (Lu et al., 2014). Outside the cell, apart from its effects on purinergic receptors, ATP acts as a positive allosteric modulator at the ionotropic $GABA_A$ receptor (Liu and Wang, 2014), and it has been reported to have both positive (Kloda et al., 2004) and negative (Ortinau et al., 2003) allosteric effects on NMDA receptor activity. The present work points to a hitherto unrecognized effect of ATP on GPCR function.

In recent years, allosteric sites on GPCRs have received much attention as potential therapeutic targets (Foster and Conn, 2017; Gentry et al., 2015). Drugs that act via allosteric sites have been recognized for their potential to be more selective and safer than orthosteric drugs, as effects of allosteric drugs may be limited by the availability of endogenous agonist (Khoury et al., 2014). The development of small, non-peptide ligands that target family B GPCRs such as PTH1R has been limited by: i) broad diffuse orthosteric binding sites on family B GPCRs; and ii) current incomplete knowledge of putative allosteric sites (Wootten et al., 2017). Among non-peptide drugs currently identified for family B GPCRs, several are allosteric modulators (de Graaf et al., 2011), whereas others may bind to sites that overlap with the relatively large orthosteric site (Carter et al., 2015).

The present studies point to a site on PTH1R or a closely associated protein that, when occupied by a suitable ligand, greatly facilitates activation of the receptor by agonists. Specifically, extracellular nucleotides such as ATP and CMP, while producing no measurable effects on their own, markedly enhance stimulation of adenylyl cyclase activity by PTH.

Comparable nucleotide effects were observed with respect to downstream CREB signaling, as well as PTH-induced changes in PTH1R-β-arrestin association. The similar nucleotide-induced changes in PTH concentration dependence with respect to cAMP production and PTH1R-β-arrestin association implies that the observed allosteric effects are independent of downstream signaling proteins, as these differ between the two pathways (*i.e.* Gs-induced adenylyl cyclase activation *vs* GPCR kinase (GRK)-dependent β-arrestin recruitment).

The effect of ATP on PTH1R signaling manifests as an increase in potency, with no observable change in maximal receptor signaling. This shift in concentration dependence and the accelerated rate of signaling in the presence of ATP is consistent with an increase in the ability of the agonist to: i) bind to the orthosteric site; and/or ii) initiate receptor activation once bound. The novel allosteric effect reported here requires the presence of an agonist, which suggests that nucleotide binding *per se* is not sufficient to appreciably activate the receptor.

In the absence of the phosphodiesterase inhibitor IBMX, cAMP levels peaked sooner with ATP regardless of PTH concentration. Although the mechanism underlying the observed earlier downward phase in cAMP time-dependence is unknown, it may reflect accelerated GRK-dependent phosphorylation of the activated receptor, subsequent β-arrestin binding (Fig. 2.11), and receptor desensitization. In addition, it is possible that elevated cAMP levels lead to the activation of phosphodiesterase, as described in other systems (Ekholm et al., 1997).

Although the present work does not directly speak to where on PTH1R extracellular nucleotides might be acting, some possibilities are suggested by functional and structural studies on PTH1R and other family B GPCRs, all of which have similar topologies and share numerous identical or conservatively substituted amino acid residues (Hollenstein et al., 2014). Moreover,

the endogenous ligands for these receptors also share structural similarities (Hollenstein et al., 2014), and thus binding and signaling mechanisms are thought to be similar within this GPCR family. In this regard, the C-terminal region of the agonist tends to bind to the extended, but kinked extracellular N-terminal region mainly via hydrophobic interactions. Contrastingly, the N-terminal peptide residues engage the exposed juxtamembrane domain and extend down into the transmembrane binding pocket, and this interaction enables G protein activation (Culhane et al., 2015; Hollenstein et al., 2014). Based on this, the comparable sensitivities to ATP of PTH (1-34), which interacts with both the N-terminal and juxtamembrane domains of PTH1R, and PTH (1-14), which interacts only with the juxtamembrane domain (Culhane et al., 2015), suggest that nucleotide effects do not require direct participation of the PTH1R extracellular N-terminal region.

Taken together, our findings establish that extracellular nucleotides increase agonist affinity, efficacy or both, and are consistent with modulation of signaling through a heretofore unrecognized allosteric mechanism at the level of the receptor or a closely associated protein. It is conceivable that negatively charged phosphate-containing molecules such as ATP and CMP could alter tertiary protein structure through interaction with basic amino acid residues or by disrupting existing interactions between acidic amino acid residues and other side chains. Physiologically, this phenomenon may account for the disparity between EC_{50} values of PTH in *in vitro* assays and the relatively low concentrations of PTH in plasma. Normal serum levels of PTH are in the sub-nanomolar range (Seshadri et al., 1985); whereas, affinities and potencies experimentally measured *in vitro* tend to be in the low-to-mid nanomolar range. *In vivo,* ATP is thought to achieve concentrations sufficient to activate P2X7 receptors (high micromolar to low millimolar), especially when ATP is released in response to mechanical stimulation or injury

(Grol et al., 2009). Thus, the phenomenon reported here may account for the synergy between mechanical loading and PTH observed *in vivo* (Gardner et al., 2007; Sugiyama et al., 2008).

Future studies using high resolution structural biology will be required to elucidate the precise binding site and mechanism of action of nucleotides on PTH1R signaling. It is possible that allostery could eventually be exploited to develop therapeutic approaches for the treatment of diseases such as osteoporosis. Mimicking the effect of nucleotides with new and existing agents could potentiate signaling through PTH1R. Depending upon the distance between the two binding sites, it may even be possible to develop bitopic ligands that interact concomitantly with both the orthosteric and allosteric sites to increase therapeutic efficacy.

2.5 References

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CHAPTER THREE

GENERAL DISCUSSION AND CONCLUSIONS

3.1 Summary and conclusions

At micromolar concentrations, extracellular nucleotides such as ATP activate a number of P2 nucleotide receptors (Burnstock, 2007). Nucleotides, at these concentrations, have been shown to potentiate PTH-induced calcium, but not cAMP signaling in a P2Y receptor-dependent manner (Kaplan et al., 1995). In contrast, high concentrations of ATP are known to potentiate other receptor signaling via low affinity P2X7 receptor-dependent and -independent mechanisms (Grol et al., 2016; Liu and Wang, 2014). In this thesis, the effect of high concentrations of extracellular ATP on PTH1R signaling was evaluated. Conclusions drawn are below.

1) Extracellular ATP, at millimolar concentrations, increases agonist potency at PTH1R. This is characterized by a leftward shift in the concentration-dependence curves determined using assays that monitored adenylyl cyclase activation and β-arrestin-1 recruitment to PTH1R. As well, concentration-dependence of extracellular ATP revealed that the effect of ATP on PTH1R saturates at concentrations of ATP at 1 mM and above.

2) Other nucleotides mimic the potentiating effect of ATP. Cytidine 5'-monophosphate (CMP), a nucleotide that lacks high energy phosphate bond and is not an agonist at P2 receptors, mimicked the potentiating effect of ATP. As well, P2 receptor antagonists failed to abolish the enhancement of PTH1R signaling to ATP, indicating that P2 receptors are not implicated with the determined potentiation.

3) ATP and CMP enhance PTH-induced activation of CREB, which is a downstream mediator of cAMP signaling; thus the potentiation of PTH1R signaling by

extracellular nucleotide propagates downstream to evoke functional effects, such as transcription modulation.

4) The molecular mechanism underlying the potentiating effect of extracellular ATP was investigated. It was determined that: i) P2 nucleotide receptors are not implicated, as CMP mimicked the potentiating effect of ATP and P2 antagonists had no significant effect on the enhancement of signaling; ii) ATP does not directly interact PTH to increase the agonist potency at PTH1R, as ATP enhanced adenylyl cyclase activation to other PTH1R agonists, such as PTHrP and PTH (1-14); iii) Certain buffer constituents (BSA and Mg^{2+}) are not implicated, as ATP enhanced PTH1R signaling in the absence of these constituents; iv) Receptor activity modifying proteins (RAMPs) – membrane proteins known to interact and regulate GPCR – are not implicated, as absence or overexpression of these proteins had no effect on the potentiating effect of ATP; v) ATP does not interact with adenylyl cyclase to increase agonist potency at PTH1R, as the rate of β-arrestin recruitment is enhanced too; vi) extracellular kinases or nucleotidases are not implicated as CMP enhanced PTH1R signalling. Therefore, ATP is thought to enhance PTH1R signaling by acting as an allosteric modulator at PTH1R or a closely associated protein.

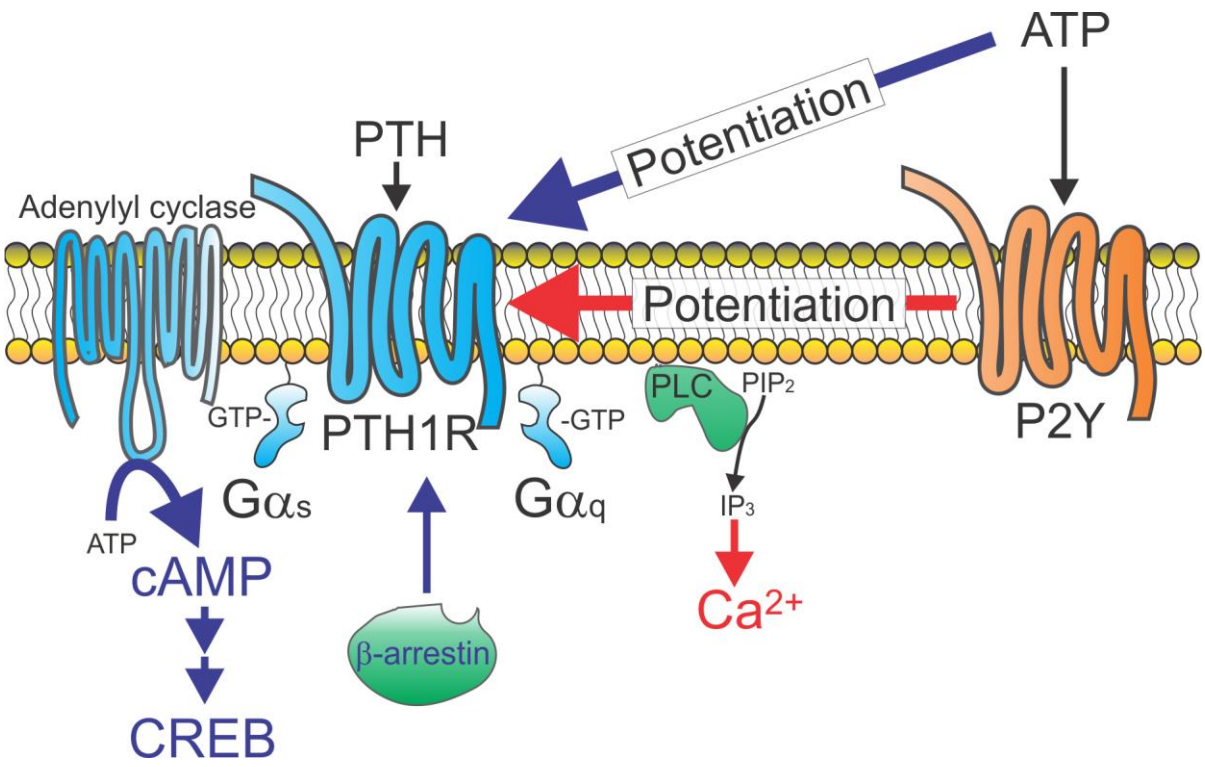

Fig. 3.1. Schematic illustrating the effect of extracellular nucleotides on PTH1R signaling. Parathyroid hormone (PTH) is an endocrine factor that activates PTH1R, which is a G protein-coupled receptor that signals through Gαs/adenylyl cyclase and Gαq/phospholipase C (PLC). Consequently, PTH1R activation results in rise in cytosolic cAMP and Ca^{2+} level. As well, activation of PTH1R results in recruitment of β-arrestin to the receptor, which leads to internalization and MAPK signaling. Activation of high-affinity P2Y receptors by extracellular nucleotides results in potentiation of PTH1R signaling, which is characterized by enhanced rise in Ca^{2+}, but not cAMP. ATP, at millimolar concentrations, potentiates cAMP signaling and β-arrestin recruitment to PTH1R, in a P2 receptor-independent manner.

3.2 Limitations of study

Many experiments performed for this thesis utilized GloSensor™ assay for measurement of cAMP levels, a luminescent protein complementation assay to monitor β-arrestin-1 recruitment to PTH1R, and a CRE-luciferase reporter assay to assess CREB activation. All three methods involved measurement of bioluminescence. The luciferase-induced bioluminescence reaction uses ATP as a substrate (Hastings, 1983); however, a number of observations argue against the possibility that increased substrate concentration was responsible for the observed potentiating effects. First, other nucleotides that are not substrates for luciferase mimic the potentiating effect of ATP. Second, ATP is highly charged and does not readily cross the plasma membrane; moreover, the concentration of extracellular ATP used in the study was less than that reported to be in the cytosol (Traut, 1994). Third, we confirmed that the potentiating effect of ATP is not dependent on the bioluminescence reaction by using an alternative assay for cAMP (the cAMP-screening system).

Most experiments involved transfection with an expression vector that encoded luciferase or fragment promoted by various promoters and enhancers. In the GloSensor™ and luminescent protein complementation assays, which monitored cytosolic cAMP level and β-arrestin-1 recruitment to PTH1R respectively, plasmids contained genes encoding intact or fragmented luciferase driven by the T7 promoter and CMV enhancer (Misawa et al., 2010; Promega Corp., 2015). Thus, transfected cells may express luciferase or fragments in an abundant manner, causing a stress to the cellular machinery. Thus, although assays used to measure cAMP level and β-arrestin recruitment are effective tools to determine dynamics of receptor signaling, they may not fully mimic true physiological conditions. In contrast, the CRE-luciferase reporter assay,

in which luciferase gene expression was enhanced by CREB, did not involve constitutive expression of luciferase. Thus, luciferase expression was controlled by CREB stimulation and this assay may better reflect physiological conditions.

PTH1R was stimulated using PTH (1-34), a 34-mer N-terminal fragment of PTH known to activate adenylyl cyclase (Habener et al., 1984). To assess whether the molecular mechanism of the potentiating effect of ATP involved interaction between agonist peptide and nucleotide, PTHrP (1-34) was used in place of PTH (Fig. 2.13). Although PTHrP and PTH are products of distinct genes, these two peptides have some protein sequence and structural similarity: eight of the first 13 amino acid residues are identical and the secondary structures are similar for the next 21 amino acids (Wysolmerski, 2012). Importantly, the first 14 residues of PTH or PTHrP are implicated with activation of PTH1R for stimulation of adenylyl cyclase (Luck et al., 1999), and the remaining portions PTH (15-34) or PTHrP (15-34) are implicated in high-affinity binding to the N-terminal loop of PTH1R (Bergwitz et al., 1996). We also reported that ATP increases agonist potency of PTH (1-14), which, on its own, binds to PTH1R with low affinity. Therefore, it is conceivable that the potentiating effect of ATP is due to interaction with the first eight residues shared among PTH and PTH1R, rather than a direct interaction with PTH1R. However, our preliminary data suggest that agonist potency at other GPCRs can be potentiated by extracellular nucleotides. Thus, it is unlikely that ATP interacts directly with agonist to increase agonist potency at PTH1R. Rather, there appears to be an allosteric interaction with PTH1R, an effect of ATP that is common for a number of GPCRs.

3.3 Significance of research and suggestions for future studies

Mechanical loading and intermittent PTH administration have been demonstrated to induce bone anabolism; moreover, these two bone anabolic stimuli have been shown to synergize (Fox et al., 1997; Klein-Nulend et al., 2012; Ma et al., 1999). Mechanical loading of cells stimulates nucleotide release (Genetos et al., 2005; Romanello et al., 2001), and extracellular nucleotides at low concentrations act via high-affinity P2 nucleotide receptors to amplify PTH1R signaling (Kaplan et al., 1995). This thesis reported that nucleotides increase agonist potency at PTH1R in a P2 receptor-independent manner, indicating that extracellular nucleotides have two distinct ways to enhance PTH1R signaling. Thus, it is possible that the molecular mechanism behind the synergistic effect of mechanical stimuli on the bone anabolic effect of PTH may, in part, be mediated by the potentiating effects of nucleotides on PTH1R signaling.

While investigating the effect of extracellular ATP or CMP on PTH-induced CREB activation, we discovered that the presence of ATP or CMP enhances CREB activation. Moreover, we found that CREB activation is enhanced by ATP significantly more than CMP (Fig. 2.13). Curiously, the potentiating effect of ATP and CMP were similar in real-time live-cell assays that monitored cAMP production and β-arrestin recruitment to PTH1R (Fig. 2.8), suggesting that extracellular ATP had yet another enhancing effect on PTH-induced CREB activation that could not be mimicked by CMP. Nucleotides are readily degraded by ectonucleotidases once released into the extracellular milieu (Yegutkin, 2014). Notably, metabolism of ATP produces additional primary messengers for both P2 and P1 nucleotide receptors (*e.g.* ADP and adenosine) (Barsotti and Ipata, 2004). The time frame of live-cell experiments was 45 and 80 min for monitoring adenylyl cyclase activity and β-arrestin

recruitment, respectively. In contrast, CRE reporter luciferase assay had a duration of 420 min. Thus, it is possible that, while performing experiments to monitor CREB activation, ATP degraded to release distinct primary messengers that may enhance PTH1R signaling. However, ATP on its own did not stimulate CREB activity. Moreover, AMP or ADP do not significantly potentiate PTH1R (data not shown), thus indicating that the greater enhancement of CREB activity by ATP than CMP is not due to ATP metabolites increasing agonist potency at PTH1R. Since it is already known that P2 receptors do not crosstalk with PTH1R to enhance cAMP signaling (Kaplan et al., 1995), it may be possible that adenosine is acting via its receptor (*i.e.* adenosine receptors) to enhance PTH1R signaling. Therefore, a future study investigating the crosstalk between adenosine receptors and PTH1R may be worthwhile. If adenosine was implicated in enhancing PTH-induced cAMP signaling and CREB activation, then the release of ATP to extracellular milieu could enhance PTH1R signaling in yet another way.

The observation that millimolar concentrations of extracellular ATP potentiates receptor signaling without implicating P2 nucleotide receptors, was first reported for an ionotropic receptor (Liu and Wang, 2014), shortly after our laboratory made the similar observation on PTH1R, a metabotropic receptor. Interestingly, both studies independently concluded that extracellular ATP acts as an positive allosteric modulator; as well, ATP concentration-dependence reported were both in millimolar range, suggesting the possibility that two reported phenomena may have a similar underlying mechanism. Thus, future studies elucidating the potentiating effect of ATP on other membrane proteins, such as other classes of metabotropic receptors (*e.g.* receptor tyrosine-kinases), may be worthwhile. As well, PTH1R is the first GPCR shown to be potentiated by extracellular ATP and it has not yet been reported whether agonist potency at other GPCRs is affected by the presence of extracellular ATP. In this regard, a future

study investigating the potentiating effect of ATP on different GPCRs may determine: 1) GPCR class-specificity, which would hint at the structure of the allosteric binding site; and 2) G protein isoform-specificity, which would identify the isoform/class of G protein affected by the potentiation. Once the receptor-specificity of the potentiating effect is elucidated, it may be possible to identify commonalities (*e.g.* common binding partners) among potentiated receptors to determine characteristics crucial to the potentiating effect of ATP.

In our preliminary studies aimed at defining the pharmacophore implicated in potentiation, we have discovered that certain phosphorylated sugars mimic the ability of ATP to increase the agonist potency of PTH1R. Because phosphate moieties are negatively charged, the putative allosteric site can be speculated to have net positive charge. Thus, identifying positively charged amino acids within the putative target (*i.e.* receptor), and performing site-directed mutagenesis to identify crucial residues may aid in locating the allosteric site. Using knowledge gained from studies investigating the pharmacophore, *in silico* docking analysis, using a software such as Galaxy7TM (Lee and Seok, 2016), can be utilized to model the interaction between the allosteric modulator (*i.e.* nucleotide) and the receptor. Then, key amino acid residues within the receptors may be identified. Thus, identifying the minimal pharmacophore for the allosteric effect of nucleotides on PTH1R signaling will aid in: 1) investigating the putative allosteric site on the receptor; 2) elucidating the effect of various pharmacophores on PTH1R conformation; and, further in future, 3) developing bitopic ligands for simultaneous allosteric-orthosteric binding.

Endogenous parathyroid hormone (PTH) is an important regulator of calcium homeostasis and administration of PTH (exogenous) is used for treatment of osteoporosis. Pharmacokinetic data, such as plasma concentrations of PTH reflecting endogenous levels or

exogenous introduction of PTH, are available for review (Ebeling et al., 1992; Fox et al., 1997; Satterwhite et al., 2010). Pertinent reports reveal a striking discrepancy in between *in vivo* and *in vitro* PTH levels, where concentrations of PTH that induce robust bone anabolic responses *in vivo* fail to induce observable signaling *in vitro*. In this regard, the plasma concentration of PTH is reported to peak at 150 ng/L or ~36 pM, after subcutaneous administration of 20 µg, an amount sufficient to induce bone anabolism (Bodenner et al., 2007; Satterwhite et al., 2010). As well, the endogenous PTH concentration averaged among non-osteoporotic individuals was reported to be ~3.2 pM (Ebeling et al., 1992). In contrast, *in vitro* studies, including those described in this thesis, report that the EC_{50} for PTH-induced adenylyl cyclase activation is ~1800 pM (Thomas et al., 2009); this value is less than that for PTH-induced calcium transients or β-arrestin recruitment (*i.e.* PTH-induced cAMP signaling is more potent than other signaling events). Therefore, the EC_{50} of PTH in *in vitro* studies is about 50-fold greater than the peak concentration *in vivo*. Using appropriate parameters, the approximate degree of adenylyl cyclase activation in response to various PTH concentrations can be estimated from the dose-response equation (Hill, 1913). If adenylyl cyclase activation to PTH at 36 pM – the peak concentration *in vivo* after subcutaneous injection of PTH (Satterwhite et al., 2010) is modeled with Hill slope of 1 (Hoare et al., 2000), this concentration would evoke ~2% maximal adenylyl cyclase activation *in vitro*. Therefore, the PTH dosage used for treatment of osteoporosis should evoke strikingly minimal signaling *in vitro*. As reported in this thesis, the presence of extracellular ATP (and possibly other phosphorylated compounds) increases agonist potency at PTH1R, and enhances functional responses. Thus, the discrepancy between *in vivo* and *in vitro* studies regarding differences in PTH concentrations may be explained by the potentiating effect of extracellular ATP.

3.4 Final remarks

Osteoporosis is a prevalent devastating condition that affects many postmenopausal females (Eastell et al., 2016). Debilitation due to fear of fracture prevents patients from performing physical activities, which induces bone anabolism; inactivity, in turn, exacerbates the condition. Investigations into purinergic signaling led to understanding that extracellular nucleotides, at least in part, are molecules responsible for the mechanism underlying exercise-induced bone anabolism (Dixon and Sims, 2000; Forwood, 2001; Genetos et al., 2005). We have demonstrated that extracellular nucleotides can enhance PTH1R, a receptor implicated with bone anabolism. Thus, it may be possible that, in future, nucleotide mimetics can be developed as an "exercise drug", which could induce various beneficial effects of exercise, such as bone mass gain, to patients that are forced to remain inactive.

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APPENDIX A

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APPENDIX B

CURRICULAM VITAE

Curriculum Vitae Brandon H. Kim

Education

2016-Present **Masters in Science, Physiology and Pharmacology**
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2012-2016 **Bachelor of Medical Sciences**
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Research Publications

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Research Experience

Sept. 2016-Present **Graduate Research Assistant**
Modulation of parathyroid hormone signaling by extracellular nucleotides
- Studies on allosteric modulation by extracellular nucleotides on parathyroid hormone receptor signaling pathway

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Drs. Peter Chidiac and Jeff Dixon

Jul. 2016-Aug. 2016 **Pre-Graduate Student**
Interaction of signaling pathways controlling osteoblast activity
- Study investigating interaction between Wnt and PTH signaling pathways using luminescence-based assay

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Sept. 2015-Apr. 2016 **Undergraduate Honours Thesis Project**
Nucleotide mediated allosteric modulation of G protein-coupled receptor
- Study on Gq protein signaling using fluorescence-based dyes on osteoblast culture

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Role of ezrin-radixin-moesin (ERM) protein in chemical induced osteoclast migration

- Study on subcellular localization of proteins implicated with migration using immunoblotting and immunofluorescence on primary rat osteoclast culture

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Signaling cascade in chemical induced osteoclast migration

- Drug inhibition study using PI3Kδ specific inhibitor on primary rat osteoclast migration induced by site-directed chemotaxis

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Laboratory Research Assistant

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Apr. 2018 **BH Kim,** A Pereverzev, SM Sims, SJ Dixon, "*Modulation of parathyroid hormone receptor 1 signaling by extracellular nucleotides*", Cardiovascular Journal Club, University of Western Ontario, London, ON, Canada

Jun. 2017 **BH Kim,** A Pereverzev, SM Sims, SJ Dixon, "*Modulation of parathyroid hormone receptor 1 signaling by extracellular nucleotides*", Canadian Connective Tissue Conference, McGill University, Montreal, QC, Canada

Jun. 2016 **BH Kim,** A Pereverzev, SM Sims, SJ Dixon, "*Extracellular ATP potentiates calcium signaling by parathyroid hormone through two distinct mechanisms*", Canadian Connective Tissue Conference, McMaster University, Hamilton, ON, Canada

Aug. 2015 **BH Kim,** CD Liao, SM Sims, SJ Dixon, "*M-CSF induces redistribution of ezrin-radixin-moesin proteins in migrating osteoclast*", Joint Motion Program Summer Seminar, University of Western Ontario, London, ON, Canada

Jun. 2015 **BH Kim,** CD Liao, SM Sims, SJ Dixon, "*M-CSF induces redistribution of*

ezrin-radixin-moesin proteins in migrating osteoclast", Canadian Connective Tissue Conference, Laval University, Quebec City, QC, Canada

Aug. 2014 **BH Kim,** BD Wheal, SM Sims, SJ Dixon, "*Role of PI3Kδ in osteoclast migration*", Joint Motion Program Summer Seminar, University of Western Ontario, London, ON, Canada

Scientific Poster Presentations

May. 2017 **BH Kim,** A Pereverzev, SM Sims, SJ Dixon, "*Modulation of parathyroid hormone receptor 1 signaling by extracellular nucleotides*", Canadian Connective Tissue Conference, University of Toronto, Toronto, ON, Canada

Nov. 2017 **BH Kim,** A Pereverzev, SM Sims, SJ Dixon, "*Modulation of parathyroid hormone receptor 1 signaling by extracellular nucleotides*", Physiology and Pharmacology Research Day, University of Western Ontario, London, ON, Canada

Mar. 2017 **BH Kim,** A Pereverzev, SM Sims, SJ Dixon, "*Modulation of parathyroid hormone receptor 1 signaling by extracellular nucleotides*", London Health Research Day, London, ON, Canada

Oct. 2016 **BH Kim,** A Pereverzev, SM Sims, SJ Dixon, "*Modulation of parathyroid hormone signaling by extracellular nucleotides*", Physiology and Pharmacology Research Day, University of Western Ontario, London, ON, Canada

Oct. 2015 **BH Kim,** CD Liao, SM Sims, SJ Dixon, "*Role of ERM proteins in M-CSF induced osteoclast migration*", Physiology and Pharmacology Research Day, University of Western Ontario, London, ON, Canada

May 2015 **BH Kim,** CD Liao, SM Sims, SJ Dixon, "*Role of ERM proteins in M-CSF induced osteoclast migration*", Bone and Joint Retreat, University of Western Ontario, London, ON, Canada

Scientific Memberships

Sept. 2016-Present **Western Bone and Joint Institute (BJI)**
Graduate Member
University Hospital B6-200, London, Canada

Apr. 2015-Aug. 2016 **Western Bone and Joint Institute (BJI)**
Undergraduate Member
University Hospital B6-200, London, Canada

Jun. 2015-Present **Canadian Connective Tissue Society**

Academic Awards and Achievements

May. 2017	**2nd Place Poster Presentation Award, Graduate Student Category ($200)** Canadian Connective Tissue Conference University of Toronto Toronto, Canada
Nov. 2017	**Poster Presentation Award ($200)** Physiology and Pharmacology Research Day University of Western Ontario London, Canada
Jun. 2017	**Oral Presentation Award ($250)** Canadian Connective Tissue Conference McGill University, QC, Canada
Sept. 2016 – Aug. 2018	**Bone and Joint Institute (BJI) Transdisciplinary Bone and Joint Training Award ($10,000/year)** University of Western Ontario London, Canada
May 2015-Aug. 2015	**Joint Motion Program (JuMP) Summer Student Research Award ($6000)** University of Western Ontario London, Canada
May 2014-Aug. 2014	**Joint Motion Program (JuMP) Summer Student Research Award ($6000)** University of Western Ontario London, Canada
2012, 2013, 2015	**Dean's Honour List** University of Western Ontario London, Canada
2008-2012	**Honour Roll** Port Credit Secondary School Mississauga, Canada
2012	**Ontario Scholar** Port Credit Secondary School Mississauga, Canada