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CYCLIC NUCLEOTIDE INACTIVATION IN OSTEOBLASTS AND OSTEOSARCOMA CELL LINES

Academic dissertation

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ABSTRACT

Ahlström M. Cyclic nucleotide inactivation in osteoblasts and osteosarcoma cell lines [dissertation]. Helsinki. University of Helsinki 2001.

The cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are crucial second messengers in the hormonal regulation of bone metabolism. The mechanisms involved in the generation of cyclic nucleotides are rather well characterized. However the mechanisms of inactivation of cAMP and cGMP in osteoblasts are poorly defined. Therefore, the main aim for this thesis was to address the question of which components and mechanisms take part in the inactivation of cyclic nucleotides in osteoblasts and how this inactivation is regulated. Both cAMP and cGMP are known to mainly be inactivated by the cyclic nucleotide phosphodiesterases (PDEs), a superfamily of enzymes divided into 11 known families, designated PDE1-11.

UMR-106 rat osteoblast-like cells were used for studying the regulation of PDE-activity by parathyroid hormone (PTH) and atrial natriuretic factor (ANF). Both hormones were potent activators of PDE-activity. The effects of PTH was mediated by a cAMP/ protein kinase A dependent mechanism, and the activated enzyme was shown to be a member of the PDE4 family. As the ANF stimulated activation of PDE activity was shown to be independent of protein kinase G, the effect of ANF is likely to be mediated by direct stimulation of PDE2 activity by cGMP binding. The main cAMP hydrolyzing enzyme family in UMR-106 cells was shown to be PDE4, whereas cGMP was hydrolyzed by PDE1, PDE2 and PDE5. In human osteoblasts, the main cAMP hydrolyzing PDE families were identified as PDE1, PDE3 and PDE7. The corresponding PDE profile of human SaOS-2 osteosarcoma cells consisted of PDE1, PDE4 and PDE7. In line with the differences in PDE-profiles, inhibition of PDE activity by PDE4 selective inhibitors rolipram and Ro 20-1724 effectively potentiated prostaglandin E_2 stimulated cAMP accumulation in UMR-106 and SaOS-2 cells, but not in human osteoblasts.

ABBREVIATIONS

AC, adenylate cyclate
ALP, alkaline phosphatase
ANF, atrial natriuretic factor
AMP, adenosine monophosphate
BAR, β-adrenergic receptor
BNP, brain natriuretic peptide
8-Br-cAMP, 8-Bromoadenosine 3',5'-cyclic monophosphate
8-Br-cGMP, 8-Bromoguanosine 3',5'-cyclic monophosphate
BSA, bovine serum albumin
cDNA, complementary deoxyribonucleic acid
CNG, cyclic nucleotide gated (channel)
cAMP, adenosine 3',5'-cyclic monophosphate
cbfa1, core binding factor alpha1
CaM, calmodulin
cGMP, cyclic guanosine monophosphate
CNP, C-type natriuretic peptide
CRE, cAMP response element
CREB, cAMP response element binding protein
EPAC, exchange protein directly activated by cAMP
FCS, foetal calf serum
GC, guanylate cyclase
GMP, guanosine 3',5' monophosphate
GTP, guanosine triphosphate
HBSS, Hank's balanced saline solution
IBMX, 3-isobutyl-1-methylxanthine
mRNA, messenger ribonucleic acid
8-MMX, 8-methoxymethyl-1-methyl-3-(2-methylpropyl) xanthine
M-CSF, macrophage colony-stimulating factor
NHOst, normal human osteoblasts
NO, nitric oxide
NOS, nitric oxide synthase
NPR, natriuretic peptide receptor
OC, osteocalcin
OPG, osteoprotegerin
PCR, polymerase chain reaction
PDE, cyclic nucleotide 3', 5' phosphodiesterase
PGE_2, prostaglandin E_2
PKA, protein kinase A
PKC, protein kinase C
PKG, protein kinase G
PTH, parathyroid hormone
PTHrp, parathyroid hormone related hormone
RANK, receptor activator of NF-κB
RANKL, receptor for activator of nuclear factor-κB ligand
RT-PCR, reverse transcriptase polymerase chain reaction
V_{max}, maximal velocity constant

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following original publications, referred to in the text by their Roman numerals (I-IV):

I. Ahlström M, Lamberg-Allardt C (1997) Rapid protein kinase A-mediated activation of cyclic AMP-phosphodiesterase by parathyroid hormone in rat UMR-106 osteoblast-like cells. J Bone Miner Res 12:172-178.
II. Ahlström M, Lamberg-Allardt C (1999) Regulation of adenosine 3', 5'-cyclic monophosphate (cAMP) accumulation in UMR-106 osteoblast-like cells: role of cAMP-phosphodiesterase and cAMP efflux. Biochem pharmacol 58:1335-1340.
III. Ahlström M, Lamberg-Allardt C (2000) Inactivation of atrial natriuretic factor-stimulated cyclic guanosine 3', 5' monophosphate (cGMP) in UMR-106 osteoblast-like cells. Biochem pharmacol 59:1133-1139.
IV. Ahlström M, Lamberg-Allardt C (2001) Differences in cyclic nucleotide phosphodiesterase profile in human osteoblasts and SaOS-2 osteosarcoma cells; role in PGE_2-stimulated cAMP accumulation (submitted).

INTRODUCTION

The remodeling of bone is a process that includes both the formation and destruction of bone tissue. The skeleton is therefore not a static tissue, but rather a metabolically active organ that undergoes continuous change in shape. Different, highly specialized cells control remodeling of bone. Three main cell types with distinct functions can be found in bone tissue. The cells responsible for bone matrix deposition, or bone formation, are the osteoblasts (Puzas 1993). The secreted extracellular matrix eventually matures to the rigid material that our bones are built of. As the osteoblast form the extracellular matrix, they become embedded in the bone material and further differentiate into another cell type, the osteocyte. Approximately 10-20% of osteoblasts become osteocytes. At the point when the osteocytes become completely embedded into the bone material, their metabolic activity decreases dramatically due to lack of nutritient diffusion. Small canals in the bone called canaliculi are at that point the only routes of nutrient and gas supply to the osteocyte (Puzas 1993).

The third type of bone cells are the osteoclasts, cells responsible for destroying, or resorbing bone. In contrast with the osteoblasts and osteocytes that origin from mesenchymal stem cells, the osteoclasts differentiate from hematopoietic progenitor cells (Mundy 1993, Aubin & Bonnelye 2000). The osteoclasts are highly specialized multinucleated cells that lie on bone surfaces. When initiating bone resorption, the osteoclasts form a sealing zone that attaches the resorbing cell to the bone matrix. Inside the sealing zone a “ruffled membrane” is formed, and the tightly sealed space between the ruffled membrane and the bone surface constitutes the osteoclast’s resorptive organelle (Mundy 1993, Väänänen et al. 2000). After the formation of the ruffled membrane, bone mineral is dissolved by acidification of the isolated microenvironment, generated by the means of an electrogenic proton pump. The organic component of bone is degraded by lysosomal proteases and the degradation products are then endocytosed at the ruffled membrane. The endocytosed vesicles are transported through the cell and released into the extracellular space at the cell’s antiresorptive surface (Salo et al. 1997, Väänänen et al. 2000, Teitelbaum 2000).

The differentiation of precursor cells into mature osteoblasts, and the functional regulation of the osteoblasts are under the control of many extracellular signals, typically hormones, such as parathyroid hormone (PTH), calcitonin and prostaglandins.

These two hormones are, together with 1,25-Dihydroxyvitamin D and estrogen, among the best known of the factors that regulate the remodeling of bone (Mundy 1993). It is well known that the intracellular signalling of both PTH and prostaglandin E_2 (PGE_2) include cyclic 3', 5-adenosine monophosphate (cAMP), a cyclic nucleotide that is present in all animal cells. The signalling cascades involving alternations in the intracellular levels of cAMP in turn affect a wide array of components in the intracellular communication of the osteoblasts, making this molecule a pivotal factor in the metabolism of bone (Partridge et al. 1994). Another cyclic nucleotide, cyclic 3', 5-guanosine monophosphate (cGMP), also participate in the hormonal signalling of animal cells. The role of cGMP in bone metabolism is not as well known as cAMP, but the effects of factors signalling through cGMP, such as natriuretic factors and nitric oxide are beginning to emerge (Hagiwara et al 1996, Suda et al. 1999, Ralston 1997, Hikiji 1997). This work was conducted to elucidate the mechanisms that inactivate cAMP and cGMP, and might provide more insight to the role of cyclic nucleotide signalling in the regulation of bone metabolism.

REVIEW OF THE LITERATURE

1. The role of osteoblast in the regulation of bone remodeling

The role of systemic factors, such as PTH and vitamin D in the remodeling process has been known for decades. Factors such as insulin-like growth factors (IGF I and IGF II) and transforming growth factor-β are known to take part in the bone remodeling at a local level. These local factors are in turn regulated by the systemic hormones that stimulate cAMP synthesis in bone cells (Canalis, 1993). The bone remodeling cycle is highly regulated by interaction between the osteoblasts and the osteoclasts. Udagawa et al. (1990) were first to note that stromal cells or their osteoblast progeny are required for the maturation of osteoclasts. In the light of recent advances in the field of bone biology, it is now evident that some newly identified local factors expressed by the osteoblast lineage have a major impact in the control of osteoclast activity. One of these factors, termed receptor for activator of nuclear factor-κB ligand (RANKL, also known as ODF, OPGL and TRANCE) is either secreted by, or expressed as a membrane bound form on the outer surface of the cell. Osteoclast precursor cells possess receptors for

RANKL that are termed RANK. It is believed that RANKL is most abundantly expressed by bone marrow stromal cells or osteoblast progenitor cells, but also to a lesser degree by mature osteoblasts (Teitelbaum, 2000). The main effect of RANKL, in concert with macrophage colony-stimulating factor (M-CSF), which is also secreted by stromal cells and osteoblast, is to promote the differentiation and the activity of osteoclasts, and consequently increase bone resorption.

Osteoblasts/stromal cells also express another factor called osteoprotegerin (OPG), which is another key regulator of osteoclast function (Teitelbaum, 2000). The effect of OPG is to bind to RANKL and compete with RANK, thus reducing the number of activated RANK receptors on the osteoclasts. As a consequence the osteoclast differentiation is blunted, and the rate of bone resorption is reduced.

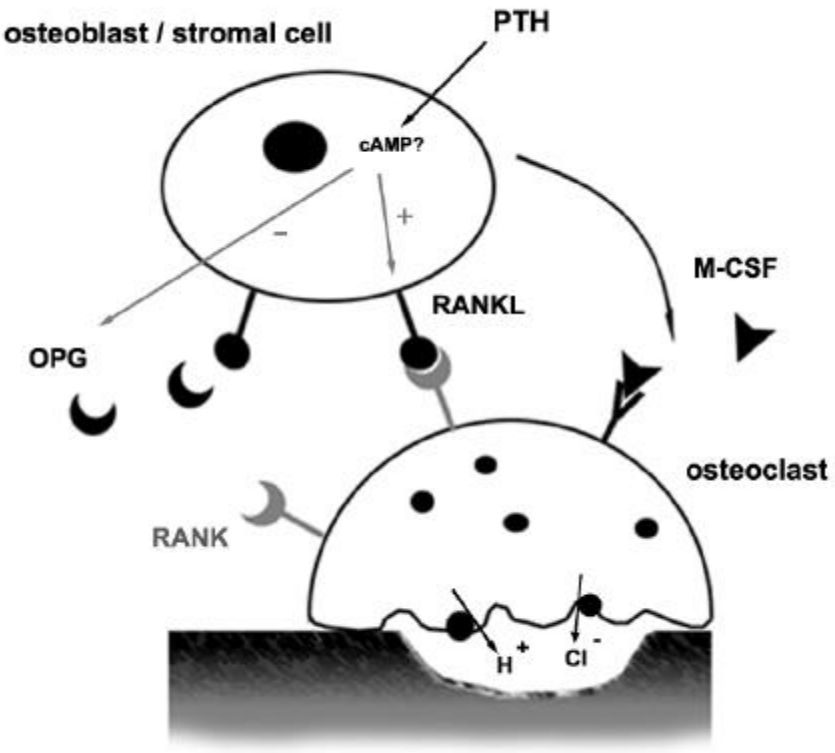

Figure 1. Relationship between osteoblasts / stromal cells and osteoclasts in the regulation of bone resorption. PTH induces the expression of RANKL, which by interacting with RANK receptors on the osteoclasts leads to increased bone resorptive activity under the apical side of the osteoclast. PTH also reduces the expression of OPG resulting in increased RANK / RANKL binding (in part adapted from Teitelbaum 2000).

In osteoblasts PTH has been shown to up-regulate the expression of RANKL and down-regulate the secretion of OPG, which could explain at least some of the catabolic effects of PTH on bone (Figure 1). In addition to resorption, the bone formation by the osteoblasts has an equally important role in the regulation of bone remodeling. Core binding factor alpha1 (cbfa1), the only known osteoblast-specific transcription factor, was recently identified (Ducy et al. 1997). It seems to function as a "master" regulator of osteoblast differentiation. Cbfa1 deficient mice develop a skeleton that is made exclusively of cartilage, which supports the hypothesis of a central role of cbfa1 as a regulator of osteoblast differentiation (Ducy et al. 2000). As no cell type autonomously decides to differentiate along a particular lineage, one of the challenges of osteoblast research has been to identify the extracellular signals that regulate the expression of cbfa1. Activation of the cAMP pathway has been shown to inhibit the DNA binding ability of cbfa1, and to inhibit the expression of cbfa1 regulated genes in an osteoblastic cell line (Tintut et al. 1999). The control of cbfa1 expression by several growth factors has also been implicated. Bone morphogenic proteins have been shown to induce, and TGF-β to inhibit cbfa1 expression *in vitro*. In addition, the gene for osteocalcin, which is a protein secreted by terminally differentiated osteoblasts, is also controlled by cbfa1 (Ducy et al. 1997). Due to its recent discovery, the regulation of cbfa1 and its effects on bone formation in osteoblast are still partly unknown. However, it seems clear that cbfa1 function is not limited to cell differentiation. Cbfa1 has been shown to regulate the level of bone matrix deposition in already differentiated osteoblasts, and one implication of these findings is that increased levels of cbfa1 transcription could be a way to prevent the appearance of osteoporosis (Karsenty 2000).

2. Molecular mechanisms and actions of the cAMP second messenger pathway

2.1 The seven-transmembrane receptors coupled to the cAMP pathway

Transmembrane receptors can be divided into three major types: G-protein-coupled receptors, enzyme-linked receptors and ion-channel linked receptors. The receptors that use cAMP as second messenger belong to the G-protein coupled seven transmembrane receptors. In order to trigger the elevation of intracellular cAMP, at least three mebrane components are required: a transmembrane receptor, a heterotrimeric G-protein

complex and adenylate cyclase (Figure 2) (Gilman 1989, Darnell et al. 1990). The archetypal transmembrane receptor proteins that involve cAMP in signalling (e.g. beta-adrenergic, thyroid stimulating hormone-, prostaglandin E_2- and parathyroid hormone receptors) consist of extracellular, transmembrane and intracellular domains. The receptor protein itself contains seven sequences of hydrophobic amino acids that span the plasma membrane seven times. The intermediate loops on the extracellular side forms the ligand binding part of the receptor, and the intracellular part contain domains which interact with G-proteins, and also contain sequences that can be phosphorylated by protein kinases, thereby altering the function of the receptor (Darnell et al. 1990). Despite common overall structure, the amino acid sequences of these receptors are usually not very similar. These sequence differences therefore determine which specific ligands that bind to the various receptors.

2.2 The G-protein complex and adenylate cyclase

In cAMP signalling, the G-protein functions as a communicator between the receptor and adenylate cyclase (AC), the enzyme responsible for the formation of cAMP from the precursor nucleotide ATP. The G-protein is composed of three peptide chains: α, β, γ. The binding of a ligand to its specific receptor leads to a conformational change, causing the receptor to bind to the G-protein. The α subunit of the G-protein, Gsα, can bind both GTP and GMP, but is in its active form only when bound to GTP. When the Gsα subunit replaces bound GMP by GTP the G-protein is dissociated into two parts: Gβ,γ and Gsα. The liberated Gsα subunit, now in its activated form, binds to adenylate cyclase, causing an activation of the enzyme with consequent synthesis of cAMP (Gilman 1989). Adenylate cyclase (AC) is a membrane bound enzyme responsible for the conversion of ATP to cAMP and pyrophosphate. The stimulatory effect of AC by hormones and neurotransmitters is mediated by the Gsα subunit of the stimulatory G-protein complex, but the G-protein complex also exists in an inhibitory form. A part of the inhibitory G-protein complex, the Giα subunit is activated in similar manner as the Gsα subunit, but instead of activating AC, the Giα subunit reduces cAMP formation by inhibiting adenylate cyclase activity. Thus, it is not solely the receptor, but also the structure and function of the G-protein complex that determines if the ligand binding causes an amplification or reduction of the cAMP signal (Gilman 1989, Darnell et al.

1990). Several other regulatory mechanisms of the AC activity have been identified. AC can be regulated by both PKA and PKC mediated phosphorylation, and some AC forms are regulated by changes in intracellular Ca^{2+} levels. After the original cloning of the mammalian adenylate cyclase gene (Krupinski et al. 1989), at least nine additional isoforms of mammalian adenylate cyclases have been identified (Sunahara et al 1996).

2.3 Protein kinase A: activation and downstream targets

The protein kinase family contains hundreds of diverse but related enzymes that regulate nearly all aspects of growth, differentiation and proliferation of eukaryotic cells. PKA was one of the first protein kinase to be discovered (Walsh et al. 1968), and is responsible for transducing the cAMP second messenger signal in cells and phosphorylates a variety of proteins. It is believed that most of the effects of cAMP are attributed to the binding of cAMP to PKA. PKA consists of four subunits of which two are regulatory and two catalytic subunits. Binding of cAMP to sites on the regulatory subunits releases the two catalytic units from the PKA complex, which become activated. The target proteins of PKA are typically components of receptors, protein kinases and other enzymes (Kemp et al. 1994, Carling et al. 1997). The activated subunits does not only phosphorylate cytoplasmic substrates, as they in their activated form are capable of entering the nucleus where they can phosphorylate proteins important for the gene transcription such as the nuclear transcription factor cAMP response element binding protein (CREB). The binding of CREB and its co-regulatory molecule CREB-binding protein (CBP) to cAMP response elements (CRE) results in changes in the transcription of the particular gene, thus altering gene expression (Taylor & Radizio-Andzelm 1994, Richards 2001). Other, more direct effects on cellular responses such as PKA dependent phosphorylation of calcium channels, cyclic nucleotide 3', 5' phosphodiesterases (PDEs) and membrane receptors are also essential parts of the cAMP signal (Sculptoreanu 1995, Sette et al. 1994, Blind et al. 1995). The activation as well as inactivation of other cell signalling cascades such as the mitogen activated protein kinases (MAPKs) in a cell specific manner has also been shown, revealing a role of PKA in signalling cross-talk (Vossler et al. 1997, Qiu et al. 2000).

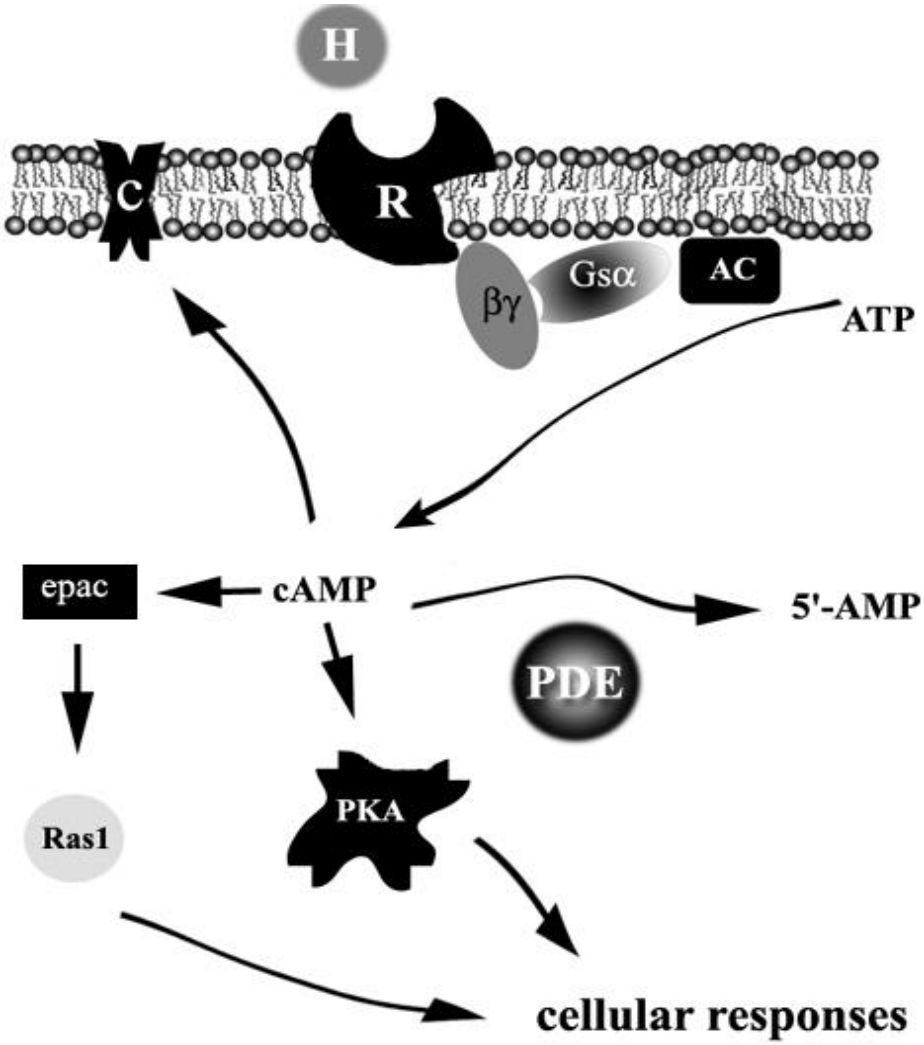

Figure 2. Activation of cAMP production and the primary target molecules for cAMP in mammalian cells. AC, adenylate cyclase; ATP, adenosine triphosphate; c, cyclic nucleotide-gated channel; epac, exchange protein directly activated by cAMP; G, G-protein complex; H, hormone; PDE, cyclic nucleotide phosphodiesterase; PKA, protein kinase A; R, transmembrane receptor.

2.4 Epac: a novel target protein for cAMP

The Ras superfamily of GTPases comprises several subfamilies of small GTP-binding proteins whose functions include the control of proliferation, differentiation, and

apoptosis (programmed cell death), as well as cytoskeleton organization. Rap1 is a small, Ras-like GTPase that can be activated by least three different second messengers, namely diacylglycerol, calcium and cyclic AMP. These second messengers activate Rap1 by promoting its release of the guanine nucleotide GDP and its binding to GTP. It was recently reported that activation of Rap1 by forskolin and cAMP occurs independently of PKA. The protein responsible for the activation of Ras1 was shown to be directly activated by cAMP and was consequently named Epac (exchange protein directly activated by cAMP). This protein contains a cAMP binding site and a domain that is homologous to domains of known guanine-nucleotide-exchange factors (GEFs) for Ras and Rap1. The binding of cAMP to Epac causes a conformational change leading to the increased activity towards Rap1 (Figure 2), which in turn mediates cellular responses (de Rooij et al. 1998).

On a cellular level the cAMP elevating agent forskolin has been shown to activate Rap1 in Rat1 cells, and the cAMP-specific type 4 family PDE inhibitor rolipram potentiates this activation (McPhee et al. 2000). Probably due to the quite recent discovery of the cAMP-Epac pathway, specific physiological functions involving this signalling cascade has not yet been identified. However, thyrotropin, through the cAMP-Epac pathway has been shown to be involved in the function, differentiation, and proliferation of dog and human thyroid cells. The cAMP-Epac cascade might therefore be of importance in the regulation of thyroid function (Dremier et al. 2000).

3. Molecular mechanisms and actions of the cGMP second messenger pathway

3.1 Natriuretic peptides and particulate guanylate cyclase

The main role of the natriuretic peptides is to defend the body against excess salt and water retention and to promote vascular relaxation (Levin et al 1998). The natriuretic peptide family consists of three identified peptides: atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). All three peptides bind to membrane bound receptors, the natriuretic peptide receptors (NPR). Three subtypes of the NPR have been identified by molecular cloning techniques. These are the NPR-A NPR-B and NPR-C (Levin et al. 1998). The NPR-A and NPR-B are coupled to intrinsic guanylate cyclase and binding of the natriuretic peptide to the

receptor leads to increased intracellular cGMP by inducing the conversion of GTP to cGMP (Figure 3). Binding of natriuretic peptide to NPR-C does not increase cGMP, because it lacks intrinsic GC, and their function are considered to be "clearance receptors" that metabolically remove natriuretic peptides, thus reducing the natriuretic peptide effects. The C-receptor is internalized after ligand binding and the ligand is enzymatically cleaved, after which the C-receptor returns to the cell surface (Waldman & Murad 1989, Anand-Srivastava and Trachte 1993, Levin et al. 1998). NPR-A binds both ANF and BNP, with preference for ANF. NPR-B, although structurally similar to NPR-A, binds CNP with high affinity, and is probably not a natural receptor for ANF and BNP. The gene for ANF codes a precursor protein of 126 amino acids, which is later cleaved into a 98 amino acid and a 28 amino acid carboxy-terminal fragment of which the latter is the biologically active form of ANF (Levin et al. 1998). ANF treatment results in rapid accumulation of cGMP in several studied tissues and target cells, and cell surface receptors for ANF has been identified in smooth muscle cells, renal membranes and pituitary cells (Koller & Goeddel 1992, Levin et al. 1998). ANF has been shown to activate cGMP-stimulated cAMP-phosphodiesterase activity, which leads to decreased cAMP followed by decreased steroidogenesis in adrenal glomerulosa cells, thus linking the cAMP and cGMP signalling pathways (MacFarland et al. 1991). The biologically active form of CNP is a 22-amino-acid peptide, structurally related to but genetically distinct from ANF and BNP. CNP is widely present in the rat, mouse, and human kidney and in the human central nervous system (Dean et al. 1994, Totsune et al. 1994). High CNP tissue concentrations also occur in the anterior pituitary, where it is a highly potent stimulator of cGMP production, and probably plays a role as an autocrine regulator of gonadotropes (McArdle et al 1994).

3.2 Nitric oxide and soluble guanylate cyclase

In addition to the activation of particulate GC by natriuretic peptides, cGMP is generated by the action of soluble GC (sGC, Figure 3). Soluble guanylate cyclase is a protein found in the cytosolic fraction of virtually all mammalian cells. It was originally purified from bovine lung, and was shown to exist as a heterodimer, of α- and β-subunits. The central part contains sequences that mediate the dimerization of the α- and β-monomers. The conversion of GTP to cGMP takes place at the C-terminal, catalytic

domain of sGC (Hobbs 1997). The acceptor molecule that triggers the cGMP synthesis of soluble GC is nitric oxide (NO). NO is synthesized from the amino acid L-arginine by the action of an intracellular soluble enzyme nitric oxide synthase (NOS), which exists as a neuronal form (nNOS), an endothelial form (ecNOS) and an inducible form (iNOS). Since its discovery NO has emerged as a mediator in several tissues and organ systems. It is involved in neurotransmission, maintenance of vascular smooth muscle tone, and immunological processes. A role of nitric oxide is also evident in a variety of pathological states such as septic shock and asthma (Moncada et al. 1991, Xu & Liu 1998).

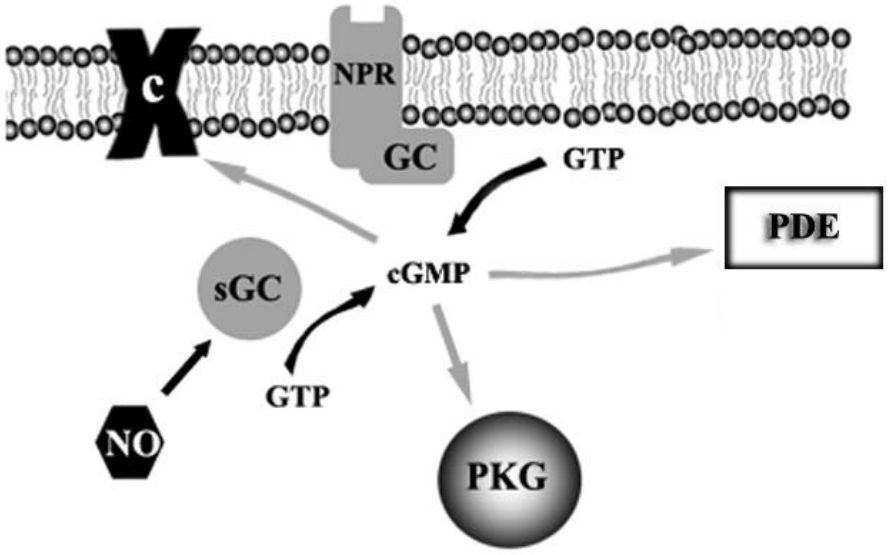

Figure 3. The formation and main target molecules of cGMP. Natriuretic peptide receptors (NPR) activate intrinsic guanylate cyclase (GC) upon receptor binding followed by cGMP production from the precursor GTP. Soluble GC, activated by nitric oxide (NO) also catalyzes the production of cGMP. Three known cGMP target molecules, protein kinase G (PKG), cyclic nucleotide phosphodiesterase (PDE) and cyclic nucleotide gated-channels (c) may be activated, leading to initiation of cellular responses.

3.3 Target molecules of cGMP

The principal targets of cGMP are: (1) cGMP-dependent protein kinases (PKG), (2) cyclic nucleotide phosphodiesterases (PDEs, separately reviewed below), (3) cyclic nucleotide-gated cation channels (CNG-channels, separately reviewed below). PKG is

likely to be the key receptor molecule in most cellular processes involving cGMP. Two genes coding for an α- and β-isoform of PKG have been described. These isoforms have regulatory domains fused to their catalytic domain. Activation of the enzyme by cGMP leads to autophosphorylation, rendering the enzyme into its active state. Activated PKG affects cellular metabolism by phosphorylating a wide range of target proteins (Kemp et al. 1994).

Of the PDEs, it is mainly the PDE2 families that are targets of cGMP. PDE2 has binding sites at the regulatory domains of the enzymes and the binding to these sites increases affinity, and results in upregulation of both cAMP and cGMP hydrolysis (Vaandrager & Jonge 1996, Conti et al. 1995, Beavo 1995). cGMP also potently regulates the cAMP-PDE activity of PDE3, but this is due to the higher affinity of cGMP for the catalytic site of PDE3. Therefore cGMP is rather a substrate than a regulatory factor for PDE3 as its effect is due to competition with cAMP. A high-affinity noncatalytic cGMP binding site has been identified on PDE5, but the binding is at least not directly associated with changes in affinity or hydrolytic activity. However, it has been suggested that binding of cGMP to the noncatalytic site transforms PDE5 into a good substrate for PKG mediated phosphorylation (Beavo 1995).

4. Cyclic nucleotide-gated channels

It has been proposed that cAMP and cGMP elicit some of their effects through direct gating of Ca^{2+} -permeable ion channels that are termed cyclic nucleotide-gated (CNG) channels (Broillet & Firestein 1999). CNG-channels are heterotrimeric proteins that are directly opened by the binding of cAMP or cGMP. The activation of the channels begins with the binding of cyclic nucleotide to a domain in the carboxyl terminal region. This binding, in turn, produces an induced fit of the protein that involves a movement of the C-helix portion of the binding domain. The induced fit of the binding domain is coupled to an allosteric conformational change opening the channel pore and allowing the influx of Na^{+} and Ca^{2+} (Zagotta 1996, Biel et al. 1998).

Most functional CNG-channels have been confined to photoreceptors and olfactory epithelium, in which CNG channels are abundant and easy to study. In vertebrate olfactory sensory neurons, the synthesis of cAMP is coupled to the odorant sensory signal. CNGs are activated by binding of cAMP and conduct a depolarizing

receptor current that leads to electrical excitation of the neuron. Photoreceptor CNG-channels have been known for decades and their important role in vision is well studied. In the dark, the cGMP levels are high due to low PDE6 activity, and the CNG-channels are consequently in opened state allowing influx of Ca^{2+}. In the light, PDE6 is activated and reduces the intracellular cGMP, thus closing the CNG-channels followed by a decrease in cGMP levels. The widespread distribution of CNG channels in the brain but also in other tissues throughout the body has only recently been recognized. cGMP-gated channels have been identified in olfactory neuroepithelium, heart and nephrons, where they might fulfil various physiological functions (McCoy et al. 1995, Vandorpe et al. 1997, Kingston et al. 1999, Broillet & Firestein 1999).

5. The cyclic nucleotide phosphodiesterases: general function and structure

The molecular machinery that is responsible of the formation of the cyclic nucleotides has been well characterized as compared to the mechanisms that inactivate the cyclic nucleotides. Phosphodiesterase activity was first described by Robinson et al. (1971), shortly after the discovery of cAMP and cGMP. In the last few years, there has been an increasing interest in the field of cyclic nucleotide inactivation by the PDEs. The PDEs hydrolyse the 3’ phosphoester bond of cAMP and cGMP to their respective biologically inactive forms 5'-AMP and 5'-GMP. This seemingly simple task has lately emerged as an important part of the regulation of different signal transduction pathways. The main tasks of the PDEs are considered to be termination of hormonal stimulation, to play a role in integrating different signalling pathways, and to serve as effectors in signal transduction (Conti 1991, Conti et al. 1995). The role of PDEs in hormonal regulation of the cell, in cross-talk between different signalling pathways, in control of the proliferation and differentiation of cells of a variable origin has been the focus in an impressive number of recent studies (Conti et al. 1995, Beavo 1995, Dousa 1999, Conti 2000).

Development of isozyme selective drugs and the use of molecular biological tools have revealed the full complexity of the PDEs; currently over 30 gene products, clustered to 11 gene families has been found in mammalian genomes (Conti et al. 1995, Guipponi et al. 1998, Dousa 1999, Soderling et al. 1999, Fujishige et al. 1999). Many products of these genes seem to be more or less tissue specific and new roles of the

PDEs in different aspects of cellular biology emerge frequently. The described tissue specificity of the PDEs has introduced the PDEs as possible target molecules for the treatment of several disorders. This, recently acknowledged potential role of PDE inhibitors as therapeutic agents, has further increased the interest in the PDEs. PDE inhibitors are considered as promising candidates for the treatment of a wide range of diseases. A number of drugs are in clinical trials for asthma, and sildenafil citrate (Viagra®) has become the first selective phosphodiesterase inhibitor to be approved by the US Food and Drug Association (Torphy 1998, Perry & Higgs 1998). Possible future applications in the treatment of cardiac disease, depression, multiple sclerosis and other autoimmune diseases have been suggested (Beavo 1995, Ekholm et al. 1997, Perry & Higgs 1998, Conti 2000). The development of inhibitors with higher selectivity and affinity towards the targeted PDEs would decrease side effects and increase the potency of such drugs. This is exemplified by sildenafil citrate, which is approximately 240-times more potent in inhibiting PDE5 than zaprinast, a traditional PDE5 inhibitor (Ballard et al. 1998). The use of short synthetic oligonucleotides (anti-sense oligonucleotides) to control gene expression is another promising therapeutic approach that could be applied to PDE inhibition (Ma et al. 2000). At an experimental level, anti-sense oligonucleotides have been applied in inhibiting PDE activity. Anti-sense oligonucleotides were used to selectively inhibit PDE7 expression in T cells resulting in reduced rate of proliferation. Thus, inhibition of PDE7 could be an approach in treating T cell-dependent disorders (Li et al 1999).

The molecular composition of PDEs present in mammalian cells is basically similar. They have a conserved region that corresponds to the catalytic domain where the hydrolyzation of cAMP and cGMP takes place. The amino terminal part contains protein interaction domains as well as binding sites for small molecules such as cyclic nucleotides. In addition, phosphorylation domains that control the catalytic function have been mapped at the amino terminal part of most PDEs. The carboxyl terminal domains may be involved in dimerization, or may function as a regulatory domain being a target for phosphorylation (Conti et al. 1995, Conti 2000). The currently used nomenclature of the PDE isozymes is described in figure 4.

PDE family	subtype (gene)	isoform (splice variant)
–	–	–
PDE1	**PDE1A**	**PDE1A2**

Figure 4. Currently used nomenclature of the PDEs according to Beavo et al. 1994, demonstrated with PDE1A2 as an example.

5.1 Calcium / calmodulin-stimulated phosphodiesterases, the PDE1 family

The PDE1 family of isozymes is perhaps the most intensively studied and best known of the multiple PDE families. The activity of PDE1 is dependent on the intracellular concentration of Ca^{2+} and calmodulin (CaM). CaM is a small protein that mediates many effects of Ca^{2+} in eukaryotic cells. Apparently the binding of the Ca^{2+}/CaM-complex to regulatory domains causes a conformational change of the enzyme that allows the displacement of an inhibitory domain from the catalytic site, leading to the activation of PDE1 (Sonnenburg et al. 1998). A tenfold stimulation of the PDE activity by Ca^{2+}/CaM binding is common. The rapid changes that take place in intracellular Ca^{2+} after stimulation by calciotropic factors can therefore regulate the PDE activity and consequently the intracellular concentration of cyclic nucleotides.

Three different PDE1 genes, PDE1A, PDE1B and PDE1C, and 9 splice products of these genes have been identified, making PDE1 one of the more diverse of the PDE families. Of interest is to note that the different PDE1 families have different affinity for Ca^{2+}/CaM. CaM can activate the splice product PDE1A1 over ten times more potently than PDE1A2, indicating that splicing is a means to regulate sensitivity to Ca^{2+}/CaM. A difference in the Ca^{2+}/CaM affinity of PDE1B and PDE1C has also been reported (Conti 2000). The products of the three different genes are also distinguished by different affinity for cAMP and cGMP. PDE1A and PDE1B isotypes have a higher affinity for cGMP than for cAMP, while some of the PDE1C subtypes have similar affinities for both nucleotides (Dousa 1995, Loughney et al 1996). In addition of being regulated by Ca^{2+}/CaM, the PDE1 isozymes are modulated by PKA- CaM-dependent protein kinase II- and by PKC mediated phosphorylation (Hashimoto et al. 1989, Spence et al. 1995,

Kakkar et al 1999). The PDE1 isozymes are therefore potential sites of cross-talk between these kinase signalling pathways.

PDE1 isoforms are expressed in the central nervous system, heart and kidney but are abundant in most tissue types. Due to the wide tissue distribution, it is not surprising that this PDE family plays a central role in many physiological and pathophysiological functions. The function of PDE1 in the pathogenesis of certain neurological conditions, such as Parkinson's disease is a central area of investigation (Kakkar et al. 1999). Various reports have indicated that PDE1 levels are elevated in tumours (Thompson et al 1980, Wei et al. 1983, Hickie et al. 1992). Inhibition of PDE1 by both PDE-inhibitors and anti-sense oligonucleotides has been shown to induce apoptosis in human leukemic cells, and the use of this mechanism to promote death of leukemic cells has been suggested (Jiang et al. 1996).

5.2 cGMP-stimulated phosphodiesterases, the PDE2 family

A PDE activity stimulated by low concentrations of cGMP was first described by Russell et al. (1973). PDE2 seems to be one of the less diverse PDE families: only one gene and three products have thus far been identified, coding for three isoforms (PDE2A1-2A3). PDE2 has two high-affinity non-catalytic binding sites for cGMP. Binding of cGMP to these sites causes an allosteric change in the catalytic domain and the activity of both cAMP and cGMP hydrolysis is stimulated several-fold (Conti 2000). PDE2 has a rather low affinity for both cAMP and cGMP and similar V_{max} for both nucleotides. However, it is believed that PDE2 function as a cGMP-stimulated cAMP-PDE in the intact cell (Beavo 1995). PDE2 isoforms has been found in adrenal cortex, several areas in the brain and in cardiac tissue (Meacci et al 1992, Repaske et al. 1993). PDE2 is a potential site for cross-talk between cAMP and cGMP-related signalling pathways and the physiological role of this PDE2 mediated cross-talk is diverse. Perfusion of single heart cells with cGMP activates PDE2 and the resulting decline in intracellular cAMP reduces cardiac cell contractility by decreasing the inward Ca^{2+}-current. In bovine aortic vascular endothelial cells, ANF stimulated cGMP reduces cAMP by activating PDE2. Thus, PDE2 associated cross-talk possibly

TABLE I
Summary of the PDE superfamily, general properties and selective inhibitors.

PDE family	genes described	number of isoforms	substrate	intracellular modulator	hormones / factors regulating activity	kinases involved in enzyme regulation
PDE1	1A, 1B, 1C	at least 9	cAMP/ cGMP	Ca^{2+}/CaM ↑		PKA, PKC, CaM-PK II
PDE2	2A	3	cAMP/ cGMP	cGMP ↑	natriuretic peptides, NO	
PDE3	3A, 3B	3	cAMP	cGMP ↓	insulin, glucagon, dexamethasone	PKA, unknown insulin-stimulated PK
PDE4	4A, 4B, 4C, 4D	at least 15	cAMP		FSH, PTH, TSH, β-AR agonists	PKA
PDE5	5A	2	cGMP	Zn^{2+}↑		PKG
PDE6	6A, 6B, 6G	2	cGMP		light	PKC
PDE7	7A, 7B	2	cAMP			
PDE8	8A, 8B	3	cAMP			
PDE9	9A	2	cGMP			
PDE10	10A	2	cAMP/ cGMP			
PDE11	11A	4	cAMP/ cGMP			

Abbreviations: β-AR, β-adrenergic receptor ; Ca^{2+}/CaM, calcium/calmodulin complex ; CaM-PK II, calmodulin dependent protein kinase II ; FSH, follicle stimulating hormone ; PK, protein kinase ; PKA, protein kinase A ; PKC, protein kinase C ; PTH, parathyroid hormone ; TSH, thyroid stimulating hormone. Explanations of symbols: ↑, modulator increases PDE activity; ↓, modulator decreases PDE activity. The table is adapted from Conti et al. (1995) and Dousa (1999).

function as a regulator of cardiac contraction (Hartzell & Fischmeister 1986, Kishi et al. 1994, Verde et al., 1999). PDE2 might also play a role in the function of the kidney. The activation of PDE2 by ANF stimulated cGMP has been shown to cause a fall in intracellular cAMP in glomerulosa cells of the adrenal cortex, which in turn results in a reduction of aldosterone production in these cells (MacFarland et al., 1991).

5.3 cGMP-inhibited phosphodiesterases, the PDE3 family

The PDE3 family has a high affinity for both cAMP and cGMP. However, it has an as much as 10-times lower V_{max} for cGMP, and therefore hydrolyzes mainly cAMP. The inhibitory effects of cGMP on cAMP hydrolysis are a result of competition by cGMP at the catalytic site of the enzyme (Beavo 1995). Two isogenes, PDE3A and PDE3B have been identified (Meacci et al. 1992, Taira et al 1993). In rat tissues the PDE3A subtype is located mainly in the cytosolic fraction, the PDE3B subtype seems to be limited to the particulate fraction (Liu & Maurice 1998). PDE3A has been found in smooth muscle, platelets and cardiac tissue, while PDE3B is abundant in liver and adipocytes. PDE3 can often be found abundantly in cells where cAMP and cGMP have similar effects; factors that elevate cGMP potentiate the effects of agonists that elevate cAMP (Beavo, 1995). The activity of both subtypes is modulated by serine/threonine phosphorylation. An increased activity of PDE3 trough PKA mediated phosphorylation has been shown in a variety of cells, such as platelets and adipocytes (Macphee et al. 1988, Grant et al. 1988, Gettys et al. 1988, Degerman et al. 1997). The PDE3B subtype is in addition activated by phosphorylation by insulin-dependent kinases. The antilipolytic effects of insulin have in fact been shown to be at least in part dependent on a decrease in intracellular cAMP. The decrease in cAMP seems to be caused by activation of PDE3B, which has been decribed in both rat and human adipocytes (Degerman et al. 1990, Eriksson et al. 1995, Degerman et al. 1997, Rondinone et al. 2000). Protein kinase B has been suggested as the kinase that phosphorylates PDE3B, thus activating the enzyme in rat adipocytes (Wijkander et al. 1998). PDE3 is also thought to be involved in glucose induced insulin secretion. PDE3 inhibition augments glucose-induced insulin secretion in an insulin-secreting cell line (Ahmad et al. 2000). Furthermore, arylpiperazine, which is an insulin secretagogue, has been shown to inhibit PDE activity in mouse pancreatic islet cells (Leibowitz 2000). The regulation of PDE3

activity by these mechanisms might have a major role in lipolysis, and PDE3 inhibitors could consequently function as possible therapeutic agents in treatment of diabetes.

Other well-known physiological roles of PDE3 are associated with the control of blood pressure, cardiac contraction and platelet function. In human and frog myocytes, a NO-donor that activates guanylate cyclase and initiates cGMP production was found to increase cAMP by inhibiting PDE3 (Kirstein et al. 1995). As elevated endogenous cAMP stimulates cardiac Ca^{2+}-current, it is possible that PDE3 plays a central role in the regulation of myocardial contraction. A similar mechanism might exist in platelets, where NO-donor induced cGMP seems to inhibit platelet function by increasing cAMP by inhibiting PDE3 activity (Maurice & Haslam 1990). PDE3 is one of the major isozymes in vascular smooth muscle, and inhibitors of PDE3 are potent smooth muscle relaxants. PDE3 inhibitors, such as milrinone, vesnarinone and cilostazol have been used in clinical trials in the treatment of congestive heart failure, as antithrombotic and antihypertensive agents (Yasunaga & Mase 1985, Beavo et al 1994, Beavo 1995).

5.4 cAMP-specific phosphodiesterases, the PDE4 family

PDE4 is the most diverse of the PDE families; 4 genes, and at least 15 different isoforms have been found. Perhaps because of this diversity and the promising role of PDE4 selective inhibitors as therapeutic agents, this enzyme is among the best characterized of the PDE families. PDE4 does not hydrolyse cGMP, and all isoforms have high affinity for cAMP. The activity of some PDE4 isoforms can be up-regulated by two distinct cAMP/PKA mediated mechanisms. PDE4D3 is activated by PKA mediated phosphorylation of the amino-terminal part of the enzyme (Sette et al. 1994, Conti 2000). This phosphorylation causes a rapid, transient activation of the enzyme, and increases its sensitivity to the PDE4 selective inhibitor rolipram (Sette et al. 1994, Alvarez et al. 1995). PDE4D1 and PDE4D2 are regulated in a “long-term” fashion via a cAMP mediated transcriptional activation, followed by up-regulation of *de novo* protein synhesis of these isoforms (Swinnen et al. 1989). In sertoli cells the PKA mediated “long-term” activation by FSH leads to a 100-fold increase in PDE4D mRNA and a 10-fold increase in PDE4 activity (Conti 2000). In human myometrial cells PDE4B and PDE4D are upregulated at both mRNA and protein level, and a role of these subtypes in

pregnancy has been suggested (Méhats et al. 1999). In a similar fashion as in PKA mediated activation of PDE3, the corresponding activation of PDE4 probably plays a role in the feedback-regulation of cAMP. The physiological roles of these mechanisms have been studied in PDE4D knockout mice, and PDE4D-null mice show a 30-40% decrease in growth rate during puberty, associated with a decrease in circulating IGF-1 levels. The PDE4 isozymes can be found in a variety of tissues, but the main interest has been focused in the role of PDE4 in the function of the brain, olfactory sensory transduction, and the involvement of PDE4 in inflammatory processes (Beavo, 1995). The use of PDE4 inhibitors as anti-inflammatory agents is currently being evaluated in several studies (Underwood et al. 1993, Underwood et al. 1998).

5.5 cGMP-binding phosphodiesterases, the PDE5 family

This PDE family is strictly selective for cGMP. Only one gene has been described, and it possesses both catalytic and non-catalytic binding sites for cGMP. The binding of cGMP to the non-catalytic binding sites does not directly affect the activity of the enzyme, but it apparently results in a conformational change that makes it more sensitive to phosphorylation by PKG and PKA. This phosphorylation leads to upregulation of the PDE activity (Corbin et al. 2000). PDE5 has been found abundantly in lung, platelets and smooth muscle cells. The PDE5 isozyme has recently attracted a lot of attention, as it is the target enzyme of the PDE5 selective inhibitor sildenafil citrate. The effect of sildenafil as a treatment of penile erectile dysfunction is mediated by an increase in smooth muscle cell cGMP as a result of the inhibited activity of PDE5 (Price et al. 1998).

5.6 Photoreceptor phosphodiesterases, the PDE6 family

Three different genes of the PDE6 family have been described (PDE6A-C). These PDE families have several different gene products. The PDE6 family is exclusively found in the outer segments of rods and cones of the retina in vertebrate eye. PDE6 appears to have a rather complex structure, and consist of a tetramer of one α-subunit, one β-subunit and two γ-subunits (Deterre et al. 1988). cGMP is a central molecule in vision and a key player in transducing the effects of light in rod cells. In dark-adapted rod

cells, a high level of cGMP acts to keep Na^+ channels open and the membrane depolarized. Absorption of light activates PDE6, and as a result, cGMP levels drop, closing Na^+ channels and hyperpolarizing the plasma membrane. The reduced level of Ca^{2+} activates guanylate cyclase, and the increased cGMP restores the cells to a new baseline state (Liebman et al. 1987, Darnell et al. 1990). PDE6 shares some features of the PDE5 family; substrate preference, affinity to cGMP and sensitivity towards some selective inhibitors are similar.

5.7 The PDE7 family

The PDE7 family was the first cAMP-specific PDE discovered that shows no sensitivity to rolipram and RO 20-1724 and has a very high affinity for cAMP. There are no reported selective inhibitors for PDE7, and it is present in only low levels in most tissues and cell types. This might be the reason for the relatively late discovery of PDE7. The PDE7 family was first characterized by Michaeli et al. (1993), although an unknown PDE activity, specific to cAMP, but insensitive to rolipram, had been described earlier in human T-lymphocyte cell lines by Ichimura and Kase (1993). Two PDE7 subtypes have been identified so far, PDE7A and PDE7B (Michaeli et al.1993, Hetman et al. 2000). The PDE7A1 isoform can be found in multiple tissues, but is predominantly expressed in lymphoid tissue, and a role in T-cell activation has been suggested (Li et al. 1999). PDE7A2 is mainly expressed in skeletal muscle and myocardium (Michaeli et al. 1993, Bloom et al. 1996, Han et al. 1997). PDE7A has also been found in airway epithelial cells (Fuhrmann et al. 1999), but determination of the functional role has been slow due to the lack of PDE7 selective inhibitors. The PDE7B subtype is most highly expressed in pancreas, brain, heart, skeletal muscle and liver and is relatively sensitive for the inhibitors IBMX and dipyridamole (Hetman et al. 2000, Gardner et al. 2000).

5.8 The PDE8 family

The third family of cAMP-specific PDEs identified was the PDE8 family (Fisher et al. 1998, Soderling et al. 1998). Two genes, PDE8A and PDE8B have been found. The two PDE8A isoforms identified have different tissue distribution: PDE8A1 is found in

multiple tissues, PDE8A2 mainly in testis and liver. PDE8B seems to be highly expressed in the thyroid, with only low expression in other tissues (Hayashi et al. 1998). Both PDE8A and PDE8B are insensitive to most known PDE inhibitors, including IBMX. However, the tissue-specific location of PDE8 in the thyroid makes it an interesting potential target for pharmacological manipulation of thyroid hormone levels.

5.9 The PDE9 family

The PDE9 isozyme was discovered almost simultaneously with PDE8. PDE9 is strictly a cGMP-hydrolyzing enzyme, which is insensitive for IBMX, and apparently lacks any non-catalytic binding sites for cGMP (Dousa 1999). PDE9 has been found in human brain, heart, spleen, prostate and colon (Guipponi et al. 1999). In murine tissue PDE9 is expressed prominently in the kidney and far less in other tissues (Dousa 1999). At least four splice products of this family have been found (PDE9A1-PDE9A4) with different N-terminal ends. The kinetic characterization, inhibitor sensitivity and regulatory properties of these enzymes are yet poorly defined. The PDE9A gene is located at chromosome 21, and it has been suggested that PDE9A could play a role in genetic diseases originating to this chromosome, such as trisomy 21, and a form of hereditary deafness (Guipponi et al., 1999).

5.10 The PDE10 family

The PDE10 family is a dual-substrate PDE that may regulate both cAMP and cGMP under physiological conditions. (Loughney et al. 1999, Soderling et al. 1999, Fujishige et al.1999, Kotera et al. 1999). Two isotypes have been described, PDE10A1 and PDE10A2. PDE10 is widely expressed, but is found most abundantly in heart, brain, kidney and testis. There are no PDE10 selective inhibitors developed as yet, but the non-specific inhibitor IBMX and the PDE5 selective inhibitor dipyridamole inhibits PDE10 moderately. The splice variant PDE10A2 can be phosphorylated on its N-terminal end, but it is not known how this affects the properties of the enzyme.

5.11 The PDE11 family

The most recently identified phosphodiesterase family, PDE11, is also a dual substrate PDE. It has less than 50% similarity to all other known phosphodiesterases, and has highest amino acid similarity with PDE5. Tissue distribution studies indicate that PDE11 expression occurs at highest levels in skeletal muscle, prostate, kidney, liver, pituitary, salivary glands and testis (Fawcett 2000, Hetman et al. 2000, Yuasa et al. 2000). PDE11 is expressed as three different transcripts suggesting the existence of multiple PDE11 subtypes. PDE11 is relatively sensitive towards the non-selective inhibitor IBMX and zaprinast and in particular for dipyridamole, which is generally considered a PDE5/PDE6 selective inhibitor.

6. Inactivation of cAMP and cGMP by cyclic nucleotide efflux

Although cyclic nucleotides are mainly inactivated by PDEs in most cells, the efflux of cAMP and cGMP are one potential way in which cells can inactivate cyclic nucleotides. An important role of the efflux mechanism has been implicated in the control of intracellular cAMP or cGMP in rat glial cells, bovine adrenal medullary cells, hepatocytes and platelets (Penit et al. 1974, Marley et al. 1992, Billiar et al. 1992, Wu et al. 1993). In most cells studied, the outward movement of cAMP and cGMP seems to have a relatively minor or no effect (Barber & Butcher 1981, Mercapide et al. 1999). The mechanism responsible for the outward movement of the cyclic nucleotides is not well studied, and the molecule(s) responsible for the efflux has not been identified and adequately characterized. However, the efflux has been shown not to be a simple diffusional, but rather an energy-dependent and unidirectional movement, blocked by prostaglandin A2 and by probenecid, a non-selective antagonist of anion-transport (Rindler et al. 1978, Barber & Butcher 1981, Billiar et al. 1992, Millul et al. 1996). In pig aortic smooth muscle cells the cAMP efflux rate seems to be connected with the concentration of extracellular adenosine (Fehr et al. 1990). It has also been suggested that the efflux rate of cGMP could be dependent on if cGMP is generated by the soluble or particulate form of guanylate cyclase (Mercapide et al. 1999). Treating hepatocytes with a combination of lipopolysaccarides, interferon γ, tumor necrosis factor and interleukin-1 activates the NO pathway, with a consequent

increase in cGMP. After 18 hours incubation with this combination, these cells release high levels of cGMP as compared to the intracellular cGMP levels (Billiar et al. 1992). These results are in line with a more recent report that suggests a role of cyclic nucleotide efflux mechanisms in long–term, but not in acute cyclic nucleotide accumulation (Mercapide et al. 1999).

7. Role of cyclic nucleotides in osteoblast biology

7.1 Effects on of hormones and factors involving cAMP in their signalling

7.1.1 Parathyroid hormone and parathyroid hormone related protein

Parathyroid hormone (PTH) is one of the central regulators of bone and mineral homeostasis. Its classical endocrine role is to regulate the concentration of calcium in the extracellular fluid. The hormone is secreted from the parathyroid glands in response to hypocalcemia and acts on the kidneys and bone, promoting reabsorption of calcium from the distal tubule and increasing bone resorption leading to an elevation of plasma calcium levels. Molecular cloning has revealed that the PTH receptors expressed in bone and kidneys are identical and belong to a subgroup of the seven-transmembrane receptors. A second member of the PTH family designated parathyroid hormone-related protein (PTHrP) has also been identified (Suva et al. 1987). PTHrP was first identified as a mediator of hypercalcemia of malignancy but was subsequently detected in many normal tissues including bone (Walsh et al. 1995). The widespread expression of PTHrP and low circulating physiological levels indicate that this is a paracrine factor rather than a hormone. Nevertheless, PTHrP and PTH share the same receptor and binds to the receptor with equal affinity. Consequently, the receptor has been designated the PTH/PTHrP receptor (Lanske & Kronenberg 1998, Kronenberg et al. 1998). These receptors are distinctive in the capacity for dual activation of both the cAMP/PKA and Ca^{2+} / PKC signal transduction pathways. The receptors couple to Gsα, which activates adenylyl cyclase leading to intracellular accumulation of cAMP and activation of PKA. They also couple to Gqα, which activates phospholipase C leading to the generation of phosphoinositides and activation of the PKC. This dual activation constitutes a complex system of regulation, which is likely to allow cross talk between the pathways.

By using truncated PTH fragments it has been shown that the first two N-terminal amino acids in the PTH and PTHrP molecules are important for the cAMP response. Hence, if the first two amino acids are deleted PKC is activated but stimulation of cAMP does not take place (Rixon et al. 1994). The cAMP signalling parthway has been shown to be a major mediator of PTH action on many osteoblastic responses. These include upregulation of osteocalcin, upregulation of collagenase secretion and increased synthesis of several cytokines (Heath et al 1984, Partridge et al. 1994). In addition the cAMP pathway regulates alkaline phosphatase, which is a marker of osteoblastic activity, and type I collagen synthesis (Majeska and Rodan 1981, Partridge et al. 1989, Gallagher et al. 1996). In mouse calvarial osteoblasts, rat MC3T3-E1 cells and rat calvarial osteoblast-rich cultures, PTH and other agents that increase cAMP inhibit osteoblast differentiation (Sabatini et al 1996, Koh et al. 1999).

Early clinical trials demonstrated that low doses of PTH have anabolic effects on humans (Reeve et al. 1976). The effects of PTH/PTHrp on bone have since been extensively studied in different experimental conditions using animal, organ culture and cell culture models (Canalis et al. 1989, Whitfield et al. 1995). In rats PTH has been shown to be more effective than estrogen or bisphosphonates for restoration of lost bone mass (Wronski et al. 1993). However, the effects of PTH can be either anabolic or catabolic depending on the administered dose, and if the treatment is continuous or intermittent (Canalis et al. 1989, Mosekilde et al. 1991, Hock & Gera 1992, Dempster et al. 1993, Li et al. 1995). Cortical porosity in patients with hyperparathyroidism has raised the concern that intermittent PTH given to treat osteoporotic patients may weaken cortical bone by increasing its porosity. In a recent study intermittently administered PTH treatment dose-dependently increased intracortical porosity in the humerus of cynomolgus monkey. However, the increased porosity did not have a significant detrimental effect on the mechanical properties of the bone (Burr et al. 2001). Mimicking the effects *in vivo*, PTH effects on bone cell cultures may also depend on whether the treatment is intermittent or not (Nishida et al. 1994), but as well on experimental conditions such as cell density and duration of treatment. In cells derived from human trabecular bone PTH stimulates proliferation only when the cells are cultured at high density (MacDonald et al. 1986). In mouse calvarial cells seeded at a low density, PTH (1-34) increases alkaline phosphatase (ALP) at both enzyme and mRNA levels. In contrast, when cells are seeded at a high density, PTH reduces ALP

activity and mRNA with a simultaneous decrease in osteocalcin secretion. These effects were shown to be cAMP dependent as they could be mimicked by the adenylate cyclase activator forskolin and the synthetic cAMP analogue dibutyryl-cAMP (Isogai et al. 1996). Recently Schiller et al. (1999) reported that the anabolic response of rat MC3T3-E1 osteoblastic cells depend on the time and duration of the PTH treatment. When PTH was administered before the 20th day of culture, the *in vitro* mineralization was decreased. Initiating the PTH treatment after day 20 left the mineralization unaffected. However, if the PTH treatment was started at day 20 and terminated at day 25, a 5-fold increase in the mineralization could be shown. Also using rat osteoblastic cells, Ishizuya et al (1997) showed that the response of PTH treatment on osteoblast differentiation is different depending on the time of exposure to PTH. When osteoblastic cells were intermittently exposed to PTH for the first hour of a 48-h incubation cycle and cultured for the remainder of the cycle without the hormone, osteoblast differentiation was inhibited as shown by suppressed alkaline phosphatase activity, bone nodule formation and osteocalcin secretion. By using inhibitors and stimulators of cAMP/PKA and Ca^{2+}/PKC it was demonstrated that cAMP/PKA was the major signal transduction pathway in the inhibitory action of PTH. In contrast, an intermittent exposure to PTH for the first 6 hours of a 48-hours cycle stimulated osteoblast differentiation. Both cAMP/ PKA and Ca^{2+}/PKC systems appeared to be involved cooperatively in the anabolic effect. In a mesenchymal C3H10T1/2 cell line, used as a differentiation model, PTH enhanced osteogenic development in cells at an early stage of differentiation, but not cells at a later stage of differentiation (Hollnagel et al 1997). These results suggest that osteoblasts not only respond differently depending on the dose and time of the PTH administration, but the response also seems to be dependent on the stage of differentiation of the treated cells.

In addition to the stage of differentiation and bone forming activity of individual osteoblast, also the total osteoblast number is important in determing the formation rate of bone. As the majority of the osteoblasts die by apoptosis, this process is potentially as important as the proliferation rate in determining osteoblast number. Recently it has been demonstrated that intermittent PTH treatment of mice increases bone fornation without increasing the generation of new osteoblasts (Jilka et al. 1999). Instead, the PTH treatment seems to increase the life-span of the osteoblasts by reducing their rate of apoptosis. The antiapoptotic effects of PTH has also been confirmed *in vitro* using

rodent and human osteoblasts, and the reported effect seems to be mediated by stimulation of cAMP production and activation of PKA (Jilka et al. 1999).

Insulin-like growth factor 1 (IGF-1) has been proposed as one of the mediators of the anabolic effects of PTH (Canalis et al. 1989, McCarthy et al. 1989, 1990, McCarthy et al. 1995). In cells from rat calvariae, a transient treatment by PTH (1-34) stimulated IGF-1 synthesis, while a continuous treatment was without effect. The effect of PTH on the IGF-1 production was shown to be mediated by cAMP. Simultaneously the intermittent PTH administration had a mitogenic effect and collagen synthesis was increased. Addition of IGF-1 antibody to the cultures abolished the increase in collagen synthesis, although the mitogenic effect was unaffected (Canalis et al. 1989, McCarthy et al. 1989, 1990, McCarthy et al. 1995). Recently also IGF binding protein (IGFBP-5) has been shown to be upregulated by cAMP mediated stimulation, in line with the hypothesis that the cAMP pathway and IGF-mediated anabolic effects are interrelated (Ji et al. 1999).

For over a decade, it has been known that osteoblasts secrete one or more factors in response to PTH that recruits and activates osteoclasts (McSheehy & Chambers 1986). It was only recently that RANKL was identified as an osteoclast-activating factor (Teitelbaum 2000). As mentioned earlier, RANKL expressed by stromal/osteoblastic cells and the binding of RANKL to RANK receptors on osteoclast precursors activate the maturation, and consequently the bone resorbing activity of the osteoclast. PTH has been shown to enhance RANKL and inhibit OPG expression, although it is not yet known if the cAMP signal transduction pathway is involved in this activation (Lee & Lorenzo 1999).

7.1.2 Prostaglandin E_2

The prostaglandins are metabolites of arachidonic acid that exert a wide range of local physiological effects through activation of G-protein-coupled prostaglandin receptors. All three of the known prostaglandins, PGE_2, PGF_2 and PGI_2, have been shown to be released by bone, although PGE_2 represents the major prostaglandin and seems to be functionally most relevant to bone (Rodan et al. 1981).

There are currently four subtypes of seven transmembrane domain receptors that bind and respond to PGE_2 (EP1, EP2, EP3 and EP4). These four receptor types are coupled

to either intracellular Ca^{2+} mobilization, or stimulation or inhibition of adenylate cyclase. Osteoblasts appear to express all four receptor types, but EP2 and EP4 seem to be coupled to cAMP production (Negishi et al. 1995, Suzawa et al. 2000). The functional significance of PGE_2 receptor activation in bone is diverse. Early reports demonstrated that PGE_2 potently increased bone resorption (Klein & Raisz 1970). More recently, anabolic effects of PGE_2 in rats have been described. Intraosseus injections of PGE_2 increased the formation of woven bone associated with an increase in osteoblast and osteoclast number (Yang et al. 1993). A 30 days PGE_2 treatment induced a massive increase in osteoblastic cells, and dramatically increased woven and lamellar bone formation in aged rats (Cui et al. 2001). In primary rat osteoblasts PGE_2 has been shown to induce morphologic changes and to upregulate IGF-1 (McCarthy et al. 1991, Yang et al. 1998). In addition, a recent study describes enhanced expression of RANKL by PGE_2 induced cAMP (Suzawa et al. 2000). PGE_2 also increases the formation of mineralized nodules, although the effect of PGE_2 has been shown to be independent of the cAMP pathway (Kaneki et al. 1999). Nevertheless, there are strong implications that activation of the EP receptor followed by the rise in intracellular cAMP accumulation has a role in mediating metabolic effects of PGE_2 in osteoblasts.

7.1.3 Agonists of the β-adrenergic receptors

The family of β-adrenergic receptors (BAR1, -2 and -3) are seven transmembrane receptors that mediate the physiological responses of catecholamines (Summers & Lynne 1993). The extracellular N-terminal region of these receptors contain the ligand binding sites, while the intracellular regions, interacts with the G-protein complex and has phosphorylation sites for both PKA and PKC, as well as for β–adrenergic receptor kinase (BARK). The more tissue specific BAR3 appears not to be expressed by osteoblasts. BAR1 and BAR2 in osteoblasts are coupled to Gs activation, which results in AMP accumulation, PKA phosphorylation and stimulation of the immediate early gene c-fos (Kellenberger et al. 1998). Heterodimers of c-fos with c-jun acting as the transcription factor AP-1, bind and regulate a wide variety of AP-1 responsive genes, including alkaline phosphatase, osteocalcin and collagen 1 (Stein & Lian 1993).

TABLE II

Summary of the responses to hormones and factors that activates the cAMP signalling pathway in osteoblasts and osteoblast-like cells.

hormone / agent	function	response	cell source	reference
PTH	proliferation	↑	primary mouse osteoblasts	Sabatini et al. 1996
PTH	collagen synthesis	↓	UMR-106 rat osteosarcoma	Partridge et al. 1989
PTH, PGE_2	collagenase secretion	↑	primary mouse osteoblasts	Heath et al. 1984
PTH, forskolin, Bu_2cAMP	ALP	↓[#]	primary mouse	Isoigai et al. 1996
		↑[§]	primary mouse	
PTH	ALP	↓	ROS 17/2 rat osteosarcoma	Majeska & Rodan 1982
PGE_2	RANKL expression	↑	primary mouse osteoblasts	Suzawa et al. 2000
PGE_2	PTH receptor regulation	↓	UMR-106 rat osteosarcoma	Mitchell & Goltzman 1990
PTH, PGE_2	IGF-1 synthesis	↑	primary rat osteoblasts	McCarthy et al. 1990, 1991
PTH	IGF binding protein-5	↑	primary rat osteoblasts	Ji et al. 1999
PTH	extracellular acidification rate	↑	SaOS-2 human osteosarcoma	Barrett et al. 1997
PTH, forskolin	OC	↑	ROS17/2.8 rat osteosarcoma	Noda et al. 1988
PTH, forskolin	MAPK activation	↓	UMR-106 and ROS 17/2.8	Verheijen & Defize 1995
	proliferation	↓	rat osteosarcoma cell lines	
PTH	prostaglandin synthase expression	↑	MC-3T3-E1 rat cell line	Tetradis et al. 1996
PTH, Bu_2cAMP	apoptosis	↓	primary mouse osteoblasts MG-63 human osteosarcoma	Jilka et al. 1999

Abbreviations: ALP, alkaline phosphatase: IGF, insulin-like growth factor; MAPK, mitogen activated protein kinase; OC, osteocalcin; PGE_2 ,prostaglandin E_2; PTH, parathyroid hormone; RANKL, receptor activator of NF-κB ligand. Explanations of symbols: ↑ increase, ↓ decrease, [#] cells seeded at high density, [§]cells seeded at low density.

8. Effects of hormones and factors involving cGMP in their signalling

8.1 Natriuretic factors and bone

As described above, the natriuretic peptides, atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) are a family of structurally related peptides that has been reported to regulate a variety of physiological processes in diverse tissues, including bone. ANF is made in the cells of the cardiac atrium, stored there in granules, and acts upon vascular smooth muscle and other targets to influence vascular tone and fluid and electrolyte balance. In bone cells, Fletcher et al. (1986) first identified ANF receptors in osteoblast from newborn rat calvaria and UMR-106-01 rat osteosarcoma cells. The binding of ANF to its receptor resulted in a 200-fold increase in intracellular cGMP. Later studies have revealed interesting effects of ANF, and other natriuretic peptides on bone metabolism. In isolated foetal rat calvaria ANF reduced the cAMP response to PTH and PGE_2, and also reduced PGE_2 stimulated bone resorption in the calvaria (Vargas et al. 1989). In MC3T3-E1 rat osteoblast-like cells, the synthetic cGMP analogue 8-Bromo-cGMP and cGMP produced by natriuretic peptides increased ALP activity, suggesting a direct involvement of the cGMP signalling pathway in the osteoblast differentiation (Nashida et al. 1996). In osteoblast cultures isolated from rat calvaria, the presence of a synthetic cAMP analogue, 8-Bromo-cAMP, resulted in both inhibition of alkaline phosphatase, and the formation of mineralized nodules. In contrast, 8-Bromo-cGMP, a corresponding analogue of cGMP had opposite effects: ALP activity was increased and the formation of mineralized nodules was promoted (Inoue et al. 1995). MC3T3-E1 cells have been shown to secrete and express CNP mRNA, and consequently CNP has been suggested to be a potent paracrine/autocrine modulator of osteoblastic cells (Suda et al. 1996). MC3T3-E1 cells respond to CNP by a decrease in proliferation rate, and stimulation of intracellular cGMP production by CNP increases the mRNA transcripts of collagen type 1, ALP and osteocalcin (Suda et al. 1996, Inoue et al 1996). In osteoblasts from newborn rat calvaria, both ANF and CNP reduced the proliferation rate, increased production of the osteoblast specific protein osteocalcin and also increased ALP, both at the mRNA and protein level (Hagiwara et al. 1996). Furthermore, the formation of mineralized nodules was also increased, suggesting anabolic effects of both ANF and

CNP. In line with these results, Suda et al. (1999) recently reported that CNP, and in a less pronounced way, ANF causes a decrease in proliferation rate and a slight increase in the ALP activity of ROB-C26 cells, which is a rat derived osteogenic cell line. In organ culture of mouse tibias CNP and 8-Br-cGMP significantly increased bone growth by stimulating endochondral ossification (Yasoda et al. 1998). However, CNP stimulation of 1,25-dihydroxyvitamin D_3-treated, osteoclast containing mouse bone marrow cultures was shown to stimulate the bone resorption (Holliday et al. 1995).

8.2 Nitric oxide and bone

Nitric oxide (NO) is a short lived free radical that is involved in various physiological processes in cardiovascular, immune and central nervous tissues. NO is known to activate soluble guanyl cyclase resulting in an increase of intracellular cGMP, this being the main effector mechanism of NO (Moncada et al. 1991, Moncada and Higgs 1993). While initial work focused on NO in the vascular and nervous systems, several studies in the last few years have demonstrated that also cells of other tissues, such as macrophages, hepatocytes, chondrocytes and bone marrow cells produce NO. Also bone cells produces NO and express NOS enzymes, and increasing evidence show that NO plays an important role as a paracrine and autocrine mediator of bone cell physiology (Ralston et al. 1994).

NO seems to be involved in anabolic processes mediated by mechanical strain, sex hormones and fracture healing, but it also mediates catabolic processes in response to inflammation. It has been shown that a slow and moderate release of nitric oxide stimulates the replication of primary rat osteoblasts and alkaline phosphatase activity, while a rapid release and high concentrations of NO inhibit proliferation and induce apoptosis. Both the stimulatory and apoptosis-inducing effects of NO on primary osteoblasts seem to be mediated by cGMP, since both can be abolished by inhibition of guanylate cyclase (Mancini et al. 2000). A direct action of NO on osteoblastic differentiation of mouse osteoblasts, at least in part mediated by cGMP, was demonstrated by Hikiji et al. (1997) by showing that NO donors stimulate osteocalcin mRNA and ALP. These effects were accompanied with a reduction of PGE_2 secretion, which in part could explain the anti-resorptive effects of NO that has been shown (Kasten et al. 1994). Furthermore, sodium nitroprusside, a NO donor, inhibits

resorption of fetal rat limb bones in organ culture (Stern & Diamond 1992). NO also inhibits osteoclast formation and activity but the molecular mechanisms behind these events are unknown (Ralston 1997). Mice deficient of nitric oxide synthase (NOS), the enzyme responsible of the production of NO, has been shown to have abnormalities in bone formation. Histomorphometric analysis showed that the NOS gene knockout mice had reduced femural bone volume and reduced bone formation rate (Aguirre et al. 2001).

TABLE III
Summary of the response to hormones and factors that activates the cGMP signalling pathway in osteoblasts and osteoblast-like cells.

hormone / agent	function	response	cell source	reference
ANF, CNP	ALP, OC, mineralization	↑	primary rat osteoblasts	Hagiwara et al. 1996
ANF, CNP	ALP	↑	ROB-C26 rat osteosarcoma	Suda et al. 1999
8-Br-cGMP	ALP, mineralization	↑	primary rat osteoblasts	Inoue et al. 1995
CNP	collagen type 1 ALP, OC	↑	MC3T3-E1 rat osteoblastic cells	Inoue et al. 1996
ANF, CNP 8-Br-cGMP	ALP	↑	MC3T3-E1 rat osteoblastic cells	Nashida et al. 1996
ANF, CNP	ALP proliferation	↑ ↓	ROB-C26 rat osteosarcoma	Suda et al. 1999
NO	ALP, OC	↑	mouse primary osteoblasts	Hikiji et al. 1997

Abbreviations: ALP, alkaline phosphatase; ANF, atrial natriuretic factor; CNP, C-type natriuretic peptide; NO, nitric oxide; OC, osteocalcin. Explanations of symbols: ↑ increase, ↓ decrease.

9. Effects of phosphodiesterase inhibitors on bone metabolism

Osteoporosis, which is caused by imbalance between bone resorption and formation, has become a major public health problem in the developed countries. The development of new drugs that inhibit the formation or activity of osteoclasts, or that promote bone formation is under continuous development in the pharmaceutical industry. Numerous new treatments have already been clinically used, and several new drugs have been developed during the last decade. The main treatments include estrogen replacement therapy, the use of selective estrogen receptor modulators (SERMs) and osteoclast inhibitors such as calcitonin and bisphosphonates (Linsay et al. 1976, Raisz 1997, Heikkinen et al. 1997, Genazzani & Gambacciani 2000). Clinical studies using daily intermittent dosage of PTH has also shown impressive gains in bone density in osteoporotic women and men (Reeve 1996, Lindsay et al 1997, Rodan & Martin 2000). New targets with promising potential are the recently identified RANKL, its receptor RANK, the RANKL decoy receptor OPG. These molecules and their corresponding signalling pathways provide a number of new therapeutic targets for osteoclast inhibition (Rodan & Martin 2000, Aubin & Bonnelye 2000). It is widely believed that the effects of PTH on bone is at least in part mediated by its stimulating effects on RANKL expression, thus making the PTH/cAMP signalling pathway an additional potential target for the treatment of osteoporosis.

The diversity and complexity of the PDE superfamily presents PDE inhibitors as promising agents for therapeutic intervention, in the treatment for a wide spectrum of disease states (Beavo 1995, Perry & Higgs 1998). A possible role of PDE inhibitors in the treatment of different diseases of bone has recently been suggested in several studies using rat and mouse model (Miyamoto et al. 1997, Waki et al. 1999, Kinoshita et al. 2000, Horiuchi et al. 2001). *In vitro* studies has shown that non-selective methylxanthine PDE inhibitors, IMBX and theophylline, and two PDE4 selective PDE inhibitors, Ro 20-1724 and rolipram, increased the release of Ca^{2+} and proline from new-born mouse calvaria in organ culture, indicating that these inhibitors stimulate bone resorption. The effect of the PDE inhibitors in this study was probably due to potentiation of the effects of prostaglandin, since the resorption could be abolished by the addition of indomethacin (Lerner et al. 1986, Ransjo et al. 1988). Further studies performed on isolated mouse calvarial osteoblasts showed that long-term treatment with

rolipram or with IBMX increases ALP, a marker of bone formation (Lundberg et al. 1999). In cultured rat bone marrow cells, denbufylline, IBMX and Ro 20-1724 increased the formation of mineralized nodules by up to 90 %, with a concomitant decrease in the number of active osteoclasts by up to 70 % (Miyamoto et al. 1997). XT-44, a novel PDE4 inhibitor, increased mineralized nodule formation by 250 %, with a 50 % reduction of active osteoclasts (Waki et al. 1999). *In vivo* experiments have been shown to be in line with the described results. In rats, inoculated with Walker 256/S carcinoma to induce an artificial osteoporotic state, bone loss was reduced by over 50 % after a two-week denbufylline treatment (Miyamoto et al. 1997). Similar results were obtained by XT-44, which recovered the bone mineral density decrease induced by neurectomy and ovariectomy in rats (Waki et al. 1999). Furthermore, Kinoshita et al. (2000) showed that a five-week treatment of mice with the PDE4 selective inhibitor rolipram or by a non-selective inhibitor, pentoxifylline significantly increased both cortical and cancellous bone mass in normal mice. In this study, histomorphometric measurements suggested that the bone mineral density increase was achieved rather by an increase in bone formation, than by a reduction in the rate of resorption. Horiuchi et al. (2001) reported that daily injections of pentoxifylline enhances bone formation on bone morphogenic protein (BMP) impregnated collagen disks, implanted into the back muscles of mice. The mechanism by which pentoxifylline enhances the BMP-induced bone formation is not known, but it seems likely that the described effects include inhibition of PDE, followed by interference in cyclic nucleotide signalling of the osteoblasts. Taken together, there is strong evidence suggesting that phosphodiesterase inhibitors increase the formation of bone, and the phosphodiesterases could therefore be potential therapeutic targets in the treatment of osteoporosis.

AIMS OF THE STUDY

The mechanisms involved in the generation of cAMP in osteoblasts are rather well characterized. The structure and function of the receptors that are connected to cAMP synthesis, such as the PTH/PTHrp receptor, and the G-protein complex coupled to adenylate cyclase are rather well defined. Many of the mechanisms of the cGMP-signalling pathway in osteoblasts are also reasonably well known. However the inactivation of both cAMP and cGMP has been largely neglected, even though it is known that intracellular cyclic nucleotide levels depend on both cyclic nucleotide generating and inactivating mechanisms. Therefore, the main aim of this thesis was to address the question of which components and mechanisms take part in the inactivation of cyclic nucleotides in osteoblasts, and how this inactivation is regulated.

The specific aims were to:

1) study the hormonal regulation of PDE activity in osteoblasts.

2) determine the role of PDEs and efflux on the inactivation of cAMP and cGMP.

3) identify the PDE families present in rat and human osteoblasts, and evaluate their role in cAMP accumulation.

4) identify the subtypes of the main PDE families present in human osteoblasts and osteosarcoma cells.

METHODS

1. Cell culture

1.1 Osteosarcoma cell lines

Two osteosarcoma cell lines, one of human- and one of rat origin were used in the study. UMR-106 rat osteosarcoma cell line was used to evaluate the regulation of PDE activity by PTH and ANF (I, III), and to study the role of cAMP and cGMP efflux (II, III). The UMR-106 cell line was originally developed by TJ Martin at the University of Sheffield. It is a clonal derivate of a transplantable osteosarcoma induced by injection of radiophosphorus (^{32}P) into a rat. UMR-106 cells have receptors, and are responsive to PTH, PGE_2 and ANF. It has been widely used as a model for studying many different aspects of osteoblast functions (Fletcher et al. 1986, Civitelli et al 1988, Zajac et al. 1992, Fang 1992, Azarani 1995). The cell line used in the present studies was purchased from American Type Tissue Collection (ATCC). UMR-106 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % FCS, 50 IU penicillin and 50 ug/mL streptomycin.

The SaOS-2 human osteosarcoma cell line has also frequently been used as an osteoblast model. It is responsive to PTH and several growth factors, such as EGF and TGFβ (Murray et al. 1987, Rodan et al. 1989, Takeuchi et al 1995). It was originally isolated and cloned from an osteosarcoma of an 11 years old female. The genome consists of 56 chromosomes, which are partly structurally rearranged. Also this cell line was purchased from ATCC. SaOS-2 cells were grown in McCoy's 5A medium, supplemented with 12.5 % FCS, 50 IU penicillin and 50 ug/mL streptomycin.

1.2 Human osteoblasts

Normal human osteoblasts (NHOst) and all cell culture reagents for NHOst cells were purchased from BioWhittaker. Two strains of NHOst cells from two different 1-year-old female donors were used. Both strains were characterized by the supplier with respect to alkaline phosphatase staining and *in vitro* mineralization. The cells were seeded at a density of 5.000 cells/cm^2 and cultured in osteoblast growth medium

(OGM), supplemented with 10% foetal calf serum (FCS), 50 μg/ml ascorbic acid, 5 mM β -glycerophosphate, 200 nM hydrocortisone 21 hemisuccinate, 0.1% gentamicin / amphotericin-B solution, at 37°C in 5% CO_2 / 95 % air atmosphere on plastic petri dishes.

2. Assay of cyclic nucleotides

2.1 Measurement of cAMP accumulation

The cells were grown on 35-mm petri dishes, or on 24-well plates. After treatments of the cells with different compounds, the cells were washed with HBSS buffer containing 118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 20 mM HEPES, 0.4 mM $CaCl_2$, pH 7.4. All incubations were carried out in HBSS buffer, at 37°. cAMP was extracted from the cells with 96 % ethanol at -18° for 3 hours. The extract was evaporated either in a vacuum oven at 37° or by N_2-gas, at 50 ° and dissolved in assay buffer (0.05 M sodium acetate, pH 6.2). To measure cAMP efflux, 100 μl samples were withdrawn from the incubation buffer. The samples were instantly boiled for 1 minute. cAMP content was determined by radioimmunoassay according to Frandsen and Krishna (1976). Succinylated samples and standards were incubated overnight with rabbit anti-cAMP antibody, using adenosine 3', 5'-cyclic phosphoric acid 2'-*O*-succinyl 3-[^{125}I] iodotyrosine methyl ester as tracer (2000 Ci /mmol). After precipitation with cold ethanol, the radioactivity of samples was counted.

2.2 Measurement of cGMP accumulation

Cells were grown to confluence on 35 mm plastic petri dishes. 20 hours before treatment with ANF and various compounds the media was changed to Dulbecco's modified Eagels medium, replacing FCS with 0.1% BSA. The cultures were washed with HBSS buffer, containing 118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 20 mM Hepes, 0.4 mM $CaCl_2$, pH 7.4). All incubations were carried out in HBSS buffer at 37° C. For measurement of intracellular cGMP, the experiments were terminated by washing twice with ice cold HBSS. The cGMP was then extracted from cells with 1 ml

96 % ethanol at -18^{o} C for 3 h. The extract was transferred to plastic tubes and evaporated in vacuum at 37^{o} C. The evaporated samples were then dissolved in 1 ml of assay buffer containing 0.05 M sodium acetate, pH 6.2. If necessary, the samples were further diluted with assay buffer, and the cGMP concentration was then determined by radio-immunoassay according to Frandsen and Krishna (1976), with [^{125}I]-cGMP as a tracer. To measure cGMP efflux, 100 µl samples were withdrawn from the incubation buffer. The samples were instantly boiled for 1 minute and assayed by radio-immunoassay.

3. Assay of PDE activity

The PDE activity was assayed essentially as described by Thompson & Appleman (1971), in an incubation buffer containing 40 mM Tris-HCl, pH 8.0, 0.1% protease inhbition cocktail (PIC) containing 0.08 mM aprotinin, 2.2 mM leupeptin, 4.0 mM bestain, 1.5 mM pepstatin A, 1.4 mM E-64 and 100 mM AEBSF, 0.05% BSA, 1 mM mercaptoethanol, 10 mM $MgCl_2$ and ^{3}H-cGMP (0.25µM) or ^{3}H-cAMP (0.5 µM). 50-100 µl sample was added to incubation buffer to give a final reaction volume of 300 µl, and the mixture was incubated at 34^{o}C for 30-60 min. The reactions were stopped by boiling tubes for 1 min. After cooling on ice, 100 µl *crotalus atrox* snake venom nucleotidase (1 mg/ml) was added, and the samples were further incubated at 34^{o}C for 10 min. 500 µl of a 1:2 slurry of AG1-X8 anion exchange resin was then added, followed by a 5 min. centrifugation at 6000 g. The radioactivity of the supernatant was counted.

4. Preparation of cell homogenates

UMR-106 cells, growing on 60 mm plastic petri dishes were treated with ANF in HBSS buffer at 37^{o}C. At the end of the experiments, the cells were washed twice with HBSS and harvested with a cell scraper into 2 ml ice-cold 40 mM Tris-HCl, pH 8.0, containing 0.1% PIC and 5 mM mercaptoethanol. The harvested cells were then homogenized by ten passages through a Teflon/glass homogenizer on ice, and

centrifuged at 800 g for 5 min. The cGMP-PDE activity of the supernatant was assayed as described above.

5. Separation of PDE activities

Five confluent cultures of either NHOst, SaOS-2 or UMR-106 cells, grown on 100 mm plastic petri dishes were washed twice with PBS buffer, harvested with a cell scraper into 6 ml ice-cold homogenization buffer (buffer A), containing 20 mM Bis-tris, pH 6.5, 5 mM mercaptoethanol, 0.1 % PIC. The harvested cells were then homogenized by ten passages on ice with a Teflon/glass homogenizer. The homogenization was repeated three times. The homogenate was then centrifuged for 20 min. at 20.000 g. Five millilitres of the supernatant was diluted with 20 mL buffer B, containing 20 mM Bis-tris, pH 6.5, 0.1 M sodium acetate, 0.02% PIC (v/v), 0.1 mM EDTA, 1 mM benzamidine and 1 mM mercaptoethanol, filtered through a 0.22 mM syringe filter, and applied to a column (5mL bed volume) of Q sepharose High Performance (Amersham Pharmacia Biotech) previously equilibrated with buffer B. After washing the column with 10 bed volumes, the PDE activities were eluted with a 0.1-1.3 M linear sodium acetate gradient in buffer B, at a flow-rate of 2.5 ml/min. Fractions of 2 ml were collected into tubes containing 50 μL 5% BSA, and assayed for either cAMP-PDE or cGMP-PDE activity as described below.

6. Identification and characterzation of the PDEs

6.1 Identification of PDE families by selective inhibitors

Several PDE families can be identified according to their sensitivity to selective inhibitors that has been developed. A list of these inhibitors, and their relative inhibitory potential of different PDE families are listed in table IV. The PDE families isolated by Q sepharose chromatography were in part identified in respect to the sensitivity towards these inhibitors. For each eluted separate PDE activity, peak fractions were pooled, and the PDE activity was measured in the presence of increasing concentrations of inhibitors (usually in the range from 0 to 64 μM). The data resulting from these assays were analyzed by non-linear regression, to obtain half-maximal

inhibiting concentration (EC_{50}) using GraphPad Prism® software. In some experiments, the PDE activities were further characterized by determing the affinity (Michaelis) constant (K_m) with the same software.

Table IV

Selective and nonselective phosphodiesterase inhibitors and their inhibitory potency towards PDE isoenzyme families.

Inhibitor	MW	IC_{50}	PDE types inhibited	reference
Cilostamide	342.4	0.07	PDE3	Verghese et al. 1995
Denbufylline	320.4	1.0	PDE4	Nicholson et al. 1989
EHNA	313.8	4	PDE2	Michie et al. 1996
IBMX	222.2	2 – 50	nonselective	Scamps et al. 1993
8-MMX	266.3	4.0	PDE1	Wells et al. 1988
Milrinone	211.2	0.30	PDE3	Harrison et al. 1986
Ro 20-1724	278.4	0.9	PDE4	Souness et al. 1991
Rolipram	275.4	0.8 – 1.0	PDE4	Underwood et al. 1994
Sildenafil	474.6	0.004	PDE5	Ballard et al. 1998
Zaprinast	271.3	0.45	PDE5	Burns et al. 1992

Abbreviations: MW, molecular weight; IC_{50}, concentration of half-maximal inhibition (expressed as μmol/L).

6.2 Identification of PDE families by sensitivity to cofactors (cGMP and calcium / calmodulin)

As some PDE families are differently affected by the addition of cGMP and Ca^{2+}/CaM, assaying the PDE activity in the presence of these cofactors is also a widely used method in the identification of PDE families. CaM, in the presence of Ca^{2+} greatly attenuates the activity of PDE1 family of PDEs, while other PDE families are practically unaffected by this cofactor (Kakkar et al. 1999). cGMP can be used to identify PDE2, which activity is greatly enhanced by low concentrations of cGMP. The activity of PDE3 is, in contrast, greatly inhibited by low concentrations of cGMP, and can consequently be used in identification of enzymes of the PDE3 family (Conti et al. 1995, Beavo 1995). 20-50 U/tube CaM, in the presence of 10 μM – 1 mM Ca^{2+} was

used to identify PDE1 activity in the PDE profiles. To identify cGMP sensitive activity, the PDE activity profile was assayed in the presence of 4μM cGMP. In some experiments, the peak fractions of the PDE profiles were subjected to PDE activity assay in the presence of 0 - 64 μM cGMP.

7. Identification of PDE subtypes by reverse-transcriptase PCR

7.1 Isolation of mRNA and cDNA synthesis

Approximately 5 x 10^6 cells, grown on 100 mm petri dishes, were washed twice with PBS. The cells were detached by trypsin-EDTA (0.5-0.02%) treatment, and centrifuged for 4 min. at 250 g. After washing the cells twice with PBS, the mRNA of the cell pellet was isolated with a mRNA purification kit (QuickPrep Micro, Amersham Pharmacia Biotech) according to the manufacturers instructions. The isolated mRNA was precipitated with 0.25 M K Acetate / ethanol and the pellet was finally resuspended in 20 μL DEPC-treated water. cDNA synthesis was performed by incubating 2 μL of the mRNA in RT reaction buffer, supplemented with 200 U/tube M-MLV reverse transcriptase, 0.01 mg/ml oligo dT primer, 0.5 mM dNTP and 20 U/tube RNAse inhibitor, in a final volume of 20μL. The reaction was performed at 37^o for 60 min. Incubations were also performed with reverse transcriptase-free tubes, for the detection of possible products originating from contaminating genomic DNA.

7.2 Detection of PDE subtypes by PCR amplification

PCR amplification of cDNA was performed in 1 x PCR buffer (Finnzymes), containing 20 U/mL Taq DNA polymerase (Finnzymes), 0.1 mM dNTP (Finnzymes), 0.4 μmol/L of each primer (except for β-actin when 1 μmol/L of primers was used) and 2μL cDNA, in a total reaction volume of 25 μL. The PCR reactions were performed in a thermal cycler, 1 min. at 94^o, 2 min. at 55^o and 2 min. at 72^o, for 40 cycles (except for β-actin, when only 34 cycles were performed). Products were then subjected to electrophoresis on ethidium bromide stained agarose gels, and photographed under UV light. In each PCR amplification, tubes containing primers for β-actin were run as positive controls and to demonstrate equal loading of cDNA.

RESULTS AND DISCUSSION

1. Hormonal regulation of PDE activity in UMR-106 cells: activation of PDE activity by parathyroid hormone and ANF

1.1 PTH stimulation of cAMP-PDE activity

In UMR-106 rat osteoblast-like cells, we demonstrated that intact PTH induces a rapid activation of the cAMP-PDE activity (I). Treating the cells with 10 nM PTH time-dependently increased the PDE activity. The activation could be seen within 2 minutes after the onset of the stimulation. Peak values were reached at 15 minutes. The acute increase in PDE activity remained elevated for at least 60 minutes. The concentration-effect of the PDE activation was also assayed. At 100 nM PTH the PDE activity was stimulated over three-fold. The minimal detected effective dose was 0.1 nM. We examined the signal transduction responsible for mediating the effects of PTH. For these experiments, the cultures were treated with activators of the cAMP/PKA pathway, and activators of the Ca^{2+}/ PKC pathway. Bu2cAMP and forskolin, which both are known to activate PKA, both mimicked the effect of PTH. Phorbol 12-myristate 13-acetate, a phorbol ester, a known activator of the PKC pathway, and A 23187, a Ca^{2+} ionophore, had no effect on the PDE activity. H-8, a synthetic inhibitor of PKA, readily abolished the stimulating effect of PTH on the PDE activity, suggesting that the route of activation of the PDE activity by PTH is mediated by PKA. Alkaline phosphatase treatment of the PTH stimulated samples were deactivated suggesting possible role of phosphorylation mechanisms in the activation of the PDE activity. Both the basal and stimulated PDE activity was inhibited by low concentrations of RO 20-1724, a PDE4 selective inhibitor, suggesting that the PDE enzyme that is activated by PTH belong to the PDE4 family. The effect of RO 20-1724 on PTH and forskolin stimulated cAMP accumulation was also studied. RO 20-1724 alone did not affect the cAMP accumulation, but potentiated the PTH stimulated accumulation 7-fold. H-8 also had an effect on both PTH and forskolin-stimulated cAMP accumulation. H-8 stimulated the cAMP accumulation about 4-fold, suggesting a functional role of the PTH stimulated PDE activation in the regulation of cAMP in intact UMR-106 cells.

A short-term activation of cAMP-PDE-activity has been shown previously in several systems. PDE2 activity is stimulated by insulin and ispoproterenol in rat adipocytes, and by insulin and prostaglandins in human platelets. In FRTL-5 rat thyroid cell line, thyroid-stimulated hormone activates a PDE4 isoform by cAMP-dependent phosphorylation (Sette et al. 1994, Conti et al. 1995). In addition to activation by phosphorylation, the PDE activity has shown to be modulated by binding of cofactors, such as cGMP, Ca^{2+}/CaM. The PDE activity can also be up regulated by transcriptional activation, triggered by hormones and growth-factors (Conti, 1995). It has been suggested that these mechanisms are important for desensitization of the cells to hormonal stimulation. In bone cells, however there is no previous data describing possible mechanisms of phosphorylation-mediated or transcriptional regulation of PDE activity by hormones.

1.2 cGMP-PDE activation by ANF mediated by cGMP

Stimulation of UMR-106 cells with 30 nM ANF caused a 3-fold increase in the PDE activity of the cells (III). The onset of the activation was rapid; peak activity was reached within 2 minutes, and the activity levels were maintained high for up to 60 minutes. Significant activation of the cGMP-PDE activity could be seen at 3 nM ANF, and at 300 nM the activity was more than 3-fold. The same treatment did not affect cAMP-PDE activity. Bu_2cGMP, a cGMP analogue that activates PKG but is a poor activator of PDE2, could not mimic the ANF-stimulated activation of cGMP-PDE. It therefore seems likely that the activation of cGMP-PDE activity is mediated by direct binding of cGMP to PDE2. This hypothesis is also supported by the detection of PDE2 activity in UMR-106 cells. The presence of similar mechanisms in a wide variety of other cell types has been described. As reviewed above, ANF has been shown to activate PDE2 activity in adrenal glomerulosa cells (MacFarland et al. 1991). ANF also decreases cAMP levels in human fibroblasts and in *xenopus* oocytes, attributable to a cGMP mediated enhanced cAMP-phosphodiesterase activity (Lee et al. 1988, Sandberg et al. 1993).

2. Efflux as a mechanism of cAMP and cGMP inactivation in UMR-106 cells

The efflux of cAMP and cGMP has been shown to require input of energy and to be an unidirectional movement, that can be potently blocked by prostaglandin A2 and by probenecid, a non-selective antagonist of anion-transport (Rindler et al. 1978, Barber & Butcher 1981, Millul et al. 1996). The effects of probenecid were evident also in UMR-106 cells: probenecid treatment abolished the cAMP and cGMP efflux by up to 90% (II, III). The blocking of the efflux of both cAMP and cGMP with probenecid had no measurable effect on intracellular levels of cyclic nucleotides. We could demonstrate that a linear relationship existed between the intracellular and extracellular levels of cAMP after stimulation of the cAMP accumulation by PTH for 10 minutes (II). These results are in line with the observations of Mercapide et al. (1999) that showed a linear relationship between intracellular and extracellular cGMP levels in aortic smooth muscle cells. In most cells studied, the outward movement of cyclic nucleotides has been shown to have a relatively minor effect on the intracellular accumulation of cAMP and cGMP (Barber & Butcher 1981, Mercapide et al. 1999). This also seems to be the case in UMR-106 cells where the efflux mechanism seems to be of minor importance within the first 30 minutes. However, our results do not exclude a role of the efflux mechanism in inactivating cAMP and cGMP over a longer time-period.

3. Identification of PDE families in UMR-106 cells

The findings that PTH activates PDE4 activity in UMR-106 cells (I) revealed the need to study the relative contribution of PDE4, and to study the possible presence of other PDE families in UMR-106 cells. Consequently chromatographic isolation and biochemical / pharmacological characterization of the PDE activities was employed to study the PDE profile of the cells. The characterization of the fractions that subsequently were isolated by anion-exchange chromatography confirmed PDE4 as the largest cAMP-PDE activity (II). The PDE4 activities separated in three different peaks, probably reflecting the presence of different PDE4 gene products present in the cells. There is no data that describes separation of PDE4 family members after chromatographic separation. However PDE1 subtypes have been shown to separate into different peaks by similar chromatographic methods (Sonnenburg et al. 1998). The PDE

subtypes in UMR-106 cells were not identified, but as some isoforms of the PDE4D subtypes have been shown to be activated by PKA (Sette et al. 1994), the cAMP-dependent induction of PDE4 activity by PTH indicates the possible presence of the PDE4D subtype in UMR-106 cells. In addition to PDE4 activity, PDE1 and PDE2 cAMP-PDE activity was detected in smaller amounts (Table V). Although the role of these activities in cAMP hydrolysis seems to be minor as compared to the PDE4 activity, PDE1, PDE2 and PDE5 were shown to be major activities hydrolyzing cGMP (Table VI). In line with the identified cGMP-PDE activities, EHNA and zaprinast which are selective inhibitors of PDE2 and PDE5, potentiated ANF stimulated cGMP accumulation in UMR-106 cells (III).

4. Identification of PDE families in human osteoblasts and osteosarcoma cells

The cAMP PDE activity of NHOst and SaOS-2 cells separated on Q-sepharose ion-exchange chromatography in three separate peaks (IV). The peaks were designated A1, A2 and A3 according to their order of elution from the anion-exchange column. The peaks were then identified by using selective inhibitors, and by assaying sensitivity for cGMP and CaM. The first peak to elute of both NHOst and SaOS-2 was identified as PDE1 by its sensitivity towards 8-MMX, a selective PDE1 inhibitor, and by the stimulating effect on PDE activity by Ca^{2+}/CaM. The second eluting cAMP-PDE peaks of NHOst and SaOS-2 cells were insensitive to the addition of high levels of several tested inhibitors. IBMX however inhibited the activity of the second peak. PDE7, the cAMP-specific, rolipram insensitive PDE family has been shown to be insensitive to most known PDE inhibitors, but relatively sensitive to IBMX (Hetman et al. 2000), suggesting that the most likely candidate for the identity of peak A2 of both NHOst and SaOS-2 cells is PDE7.

The identity of the third peak was determined by assaying sensitivity to cGMP, milrinone, rolipram and 8-MMX. The different sensitivity to cGMP and PDE inhibitors indicated a different identity of A3 peaks of NHOst and SaOS-2 cells. In NHOst cells, A3 was shown to be sensitive to milrinone, a PDE3 selective inhibitor, but unsensitive to rolipram, a PDE4 selective inhibitor. Peak A3 of SaOS-2 cells was sensitive to rolipram, but relatively insensitive to milrinone. The high sensitivity of the third NHOst peak to cGMP further suggested the NHOst peak A3 activity is a member of the PDE3

family. The only detected cGMP PDE activity in both NHOst and SaOS-2 cells consisted of two, closely eluting CaM-stimulated peaks (IV). The first of these peaks mostly hydrolyzed cGMP, but the second peak, corresponding to the fractions of the cAMP hydrolyzing peak A1, hydrolyzed both cAMP and cGMP

TABLE V

Summary of cAMP-PDE activities of the studied osteoblasts and osteosarcoma cells.

cell type	PDE1	PDE2	PDE3	PDE4	PDE7
UMR-106	+	+	-	++++	-
SaOS-2	+++	-	-	++	+
NHOst	+++	-	++	-	+

Explanation of symbols: $^{-}$ no activity detected; $^{+}$ activity less than 10 %; $^{++}$ activity less than 20 %; $^{+++}$ activity over 40 %; $^{++++}$ activity over 80% of total cAMP-PDE activity. PDE1 activity was assayed in the presence of 1 mM $CaCl_2$ / 50 U/tube CaM, and PDE2 activity was assayed with 4 μM cGMP.

TABLE VI

Summary of cGMP-PDE activities of the studied osteoblasts and osteosarcoma cells.

cell type	PDE1	PDE2	PDE3	PDE4	PDE5
UMR-106	+	+++	-	-	+++
SaOS-2	++++	-	-	-	-
NHOst	++++	-	-	-	-

Explanation of symbols: $^{-}$ no activity detected; $^{+}$ activity less than 10 %; $^{++}$ activity less than 20 %; $^{+++}$ activity over 40 %; $^{++++}$ activity over 80% of total cGMP-PDE activity. PDE1 activity was assayed in the presence of 10 μM $CaCl_2$ / 20 U/tube CaM, and PDE2 activity was assayed with 4 μM cGMP.

5. Identification of PDE1, PDE3, PDE4 and PDE7 subtypes in human osteoblasts and osteosarcoma cells

Following the biochemical identification of PDE1 as one of the major PDEs in both NHOst and SaOS-2 cells, the presence of mRNA transcripts of all the known PDE1 subtypes; PDE1A, PDE1B and PDE1C were examined by RT-PCR (IV). NHOst cells expressed mRNA transcripts for both PDE1A and PDE1C; SaOS-2 cells only PDE1C. Of the two PDE3 subtypes PDE3A and PDE3B, PDE3A mRNA was detected in both NHOst cells, and SaOS-2 cells. The rolipram insensitive / IBMX sensitive cAMP specific PDE activity detected in NHOst/SaOS-2 cells indicated the presence of PDE7. This was further examined by assaying the presence of PDE7 subtype mRNA. Two mammalian members of the PDE7 family, PDE7A and PDE7B, has thus far been identified (Michaeli et al. 1993, Hetman et al. 2000). mRNA transcripts of both the PDE7A and PDE7B subtypes were detected in NHOst and SaOS-2 cells (IV). The presence of mRNA transcripts of all known PDE4 subtypes (PDE4A-4D) were also examined, following the identification of PDE4 activity in the SaOS-2 PDE activity profile, and mRNA transcripts of the PDE4A and PDE4B subtypes were found in both NHOst cells and SaOS-2 cells (IV).

6. Effect of PDE inhibitors on PGE_2 stimulated cAMP response in human osteoblasts and SaOS-2 cells

In line with the different PDE profiles of NHOst and SaOS-2 cells, the PDE4 selective inhibitor rolipram increased PGE_2 stimulated cAMP accumulation in SaOS-2 cells much more potently than in NHOst cells (IV). The non-selective inhibitor IBMX increased the cAMP accumulation of both cells similarly, but milrinone, a PDE3 selective inhibitor, increased the cAMP accumulation in NHOst cells, but not in SaOS-2 cells. Only 1 μM rolipram was required to double the cAMP accumulation level of SaOS-2 cells. This was in contrast with milrinone, which at similar concentrations had no effects on either cell line. In NHOst cells, however, the effect of 100 μM milrinone on cAMP accumulation was significant whereas the same treatment did not affect the cAMP accumulation of SaOS-2 cells. The effects of selective PDE-inhibitors on PGE_2 stimulated cAMP accumulation in NHOst and SaOS-2 cells were in line with the

responses of UMR-106 cells. As the cAMP-PDE activity of UMR-106 cells was shown to constitute mainly of PDE4, the potentiating effects of the PDE4 selective PDE inhibitor Ro 20-1724 on PTH stimulated cAMP accumulation, and the lack of effect of milrinone seems logical (I). In intact osteoblasts, IBMX has usually been used at concentrations around 0.5 – 1.0 mM in order to achieve a cellular response (Abou-Samra et al. 1991, Fang et al. 1992, Fluhmann et al. 1998). In SaOS-2 and NHOst cells, 100 μM IBMX was sufficient to achieve a two- to three-fold increase in the PGE_2 stimulated cAMP accumulation. However, only 10 μM Ro 20-1724 was required to produce a 10-fold potentiation of PTH stimulated cAMP accumulation in UMR-106 cells (I), and only 10 μM rolipram induced a 5-fold increase in PGE_2 stimulated cAMP accumulation in SaOS-2 cells (IV). These results show the impact that selective targeting of cell specific PDE-activity has on cAMP signalling of the studied cells.

CONCLUSIONS

The results in this study suggest that rapid up-regulation of the PDE activity might be an important means by which osteoblasts regulate their response to hormonal stimulation. The role of cyclic nucleotide efflux does however not seem to be a major mechanism by which osteoblasts inactivate cyclic nucleotides.

The identification of multiple PDEs indicates that the regulation of cyclic nucleotide inactivation is a complexly regulated process in osteoblasts, and that PDE might be a significant point of cross-talk between separate signalling pathways in osteoblasts. In both normal human osteoblasts and in the malignant osteosarcoma cell line SaOS-2, PDE1 was identified as the major PDE family. The results suggest that hydrolysis by PDE1 is a central mechanism by which cyclic nucleotides are inactivated in human osteoblasts. The results also imply that calcium/calmodulin play a role in cyclic nucleotide metabolism in the studied cells. In UMR-106 rat osteosarcoma cells PDE4 and not PDE1 was the main PDE family. PDE1 was however also detected in UMR-106 cells, but only at low levels.

Another main difference in the PDE profiles was the appearant lack of PDE3 activity in UMR-106 and SaOS-2 osteosarcoma cells, while PDE3 contributed to a large part of the total PDE activity of normal human osteoblasts. The reason for this difference in the PDE profiles is not known. The osteosarcoma cell lines might

represent a differentiation stage associated with high levels of PDE4 expression, or the PDE profile might have shifted during, or after the original transformation of the cells from the normal to the malignant phenotype.

This study has shown that the effect of different PDE-inhibitors on cAMP and cGMP accumulation is dependent on the PDE-profile of the treated cells. Inhibitors of PDEs have recently emerged as a potential treatment for some malignant states of bone tissue. The identification of PDE families and subtypes in human osteoblasts might therefore benefit possible future attempts to develop PDE-based drugs that alone, or in concert with cAMP-elevating agents such as PTH and PGE_2 could be used to treat diseases such as osteoporosis.

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