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Extracellular Calcium Regulation of COX-2 Expression

Stella P. Mellas

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EXTRACELLULAR CALCIUM REGULATION
OF COX-2 EXPRESSION

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Introduction

Bone undergoes remodeling throughout life. Remodeling begins with bone resorption by osteoclasts and is followed by bone formation by osteoblasts. Resorption is said to be coupled to formation. Extracellular calcium, which inhibits osteoclastic activity while stimulating chemotaxis and mitogenesis of osteoblasts, may serve as a key coupling agent between bone formation and resorption, by inhibiting (Godwin, et al., 1996). Osteoporosis results from an imbalance in the functions of forming and resorbing cells.

When a force is applied to a tooth during orthodontic treatment, a pressure is generated along one side and a tension is generated along the other side of the root of the tooth (Storey, 1973). The bone lining the pressure side of the root undergoes resorption, allowing for movement of the root in that direction, and the tension side of the root undergoes bone formation. Therefore, resorption-formation allows for root movement through bone without a net loss or gain of total bone. Studies have shown that prostaglandins may play an important role in tooth movement (Yamasaki, et al., 1996).

In this study, we examined the effects of extracellular calcium on expression of cyclooxygenase-2 (COX-2), a limiting enzyme in the production of prostaglandins (PGs) from membrane lipids (Herschman, 1994). Prostaglandins are important in regulating bone turnover and have been shown to enhance tooth movement (Yamasaki, et al., 1996). Specifically, we examined the induction of COX-2 mRNA in osteoblastic cells by extracellular calcium and determined if the induction is transcriptionally mediated.
Background

Regulation of cell function by calcium

Calcium, a divalent cation, plays very important roles in intracellular and extracellular functions. Intracellular calcium regulates a variety of cell functions, including processes as diverse as muscle contraction, hormonal secretion, glycogen metabolism, and cell division (Seibel, et al., 1995). Calcium binding to intracellular proteins, such as calmodulin, enzymes or other cellular effector systems are activated and cell functions are accomplished.

Intracellular calcium

The intracellular free calcium concentration is about 100-200 nM, whereas the concentration within intracellular organelles is 1-20 μM. Extracellular calcium concentration is about 1.2 mM and is under rigid control. As stated, there exists a 5,000 to 10,000 fold concentration gradient and a transmembrane electrical gradient that favors movement of calcium into the negatively charged cell. Mechanisms exist that maintain this concentration gradient.

Cells remain inactive at low Ca^{2+} concentrations (10-100 nM range) (Berridge, et al, 1996). When higher concentrations of calcium are reached (500-1000 nM range), cells are activated to perform their particular functions. These changes in Ca^{2+} concentration are detected by sensors such as calmodulin and troponin C, which transduce the information into specific cellular responses. Ca^{2+} can be derived from the extracellular environment, through channels in the plasma membrane, or it can be released from internal stores, such as the endoplasmic reticulum or sarcoplasmonic
reticulum in muscle. These are known as the calcium-ON mechanisms, whereby Ca\(^{2+}\) enters the cytoplasm. Conversely, calcium-OFF mechanisms exist to remove Ca\(^{2+}\) from the cytoplasm, by pumping it either out of the cell or back into the internal stores (Berridge, et al., 1996).

Various channels are present in the plasma membrane that mediate the influx of external Ca\(^{2+}\) (Berridge, et al., 1996). Voltage-operated channels are found in excitable cells (nerve, muscle, and certain endocrine cells) and open when membrane depolarization occurs to allow extracellular Ca\(^{2+}\) to enter the cell. These channels can be regulated both by receptor-mediated and intracellular messenger (ex. cyclic AMP) systems. Receptor-operated channels are activated by binding of extracellular agonists, which are usually neurotransmitters (ex. glutamate and ATP). Calcium-release activated Ca\(^{2+}\) channels (CRAC’s) are located in the plasma membrane and inhibit Ca\(^{2+}\) entry into the cell when the ER is fully loaded. Conversely, when the ER stores are dwindling, calcium influx factor diffuses to the membrane and opens the CRAC’s to allow Ca\(^{2+}\) entry.

Intracellular calcium channels responsible for releasing Ca\(^{2+}\) from the intracellular stores are inositol 1,4,5-triphosphate receptors (InsP3Rs) and ryanodine receptors (RyRs) (Berridge, et al., 1996). InsP3 diffuses into the cytoplasm, binds to its receptor and releases stored Ca\(^{2+}\). The most important diffusible messenger for activating InsP3R and RyR is Ca\(^{2+}\) itself. The process of Ca\(^{2+}\) induced Ca\(^{2+}\) release is of fundamental importance in regulating the way in which cells mobilize Ca\(^{2+}\) from their internal stores.

Calcium OFF mechanisms function to return Ca\(^{2+}\) either to internal stores or back to the external medium (Berridge, et al., 1996). The Na\(^+\)/Ca\(^{2+}\) exchanger is found in the
plasma membrane and utilizes the energy stored in the electrochemical Na\(^+\) gradient to extrude Ca\(^{2+}\) from the cell. Plasma membrane Ca\(^{2+}\) ATPases utilize the energy of ATP to transport Ca\(^{2+}\) against the enormous electrochemical gradient that exists across the plasma membrane. The sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase pumps are found on the membranes of intracellular stores and they sequester Ca\(^{2+}\) found in the cytoplasm.

Ca\(^{2+}\) binding proteins contribute to Ca\(^{2+}\) mediated signaling, either by buffering free Ca\(^{2+}\) ions or by acting as sensors that mediate the messenger role of Ca\(^{2+}\) (Berridge, et al., 1996). Proteins capable of buffering Ca\(^{2+}\) are found in both the cytoplasm and within the ER/SR. Examples of buffering proteins include parvalbumin, calretinin and calbindin.

**Extracellular calcium and regulation of bone turnover**

Elevation of extracellular calcium has been shown to inhibit osteoclastic bone resorption and stimulate proliferation and chemotaxis of osteoblasts as well as monocytes (Godwin, et al., 1997; Sugimoto, 1993). Therefore, calcium released by bone resorption may have an important role in the coupling of bone resorption and bone formation. Although both osteoclasts and osteoblasts have calcium-sensing mechanisms, it is still controversial whether these cells express the same extracellular Ca\(^{2+}\) receptor (CaR) as that identified in parathyroid and kidney cells.
Calcium receptors in parathyroid cells

Extracellular Ca$^{2+}$ acts on parathyroid cells to regulate PTH secretion. The calcium receptor was first cloned from parathyroid cells where it functions as the principal regulator of PTH secretion (Brown, et al., 1993). This receptor is a G protein-coupled receptor whose primary physiological ligand is extracellular Ca$^{2+}$ (Nemeth, 1996). This cell surface Ca$^{2+}$ receptor (CaR) enables parathyroid cells to detect and respond to small changes in the concentration of extracellular Ca$^{2+}$. Extracellular calcium acting on the CaR increases the activity of phospholipase C, thereby producing inositol 1,4,5-triphosphate and mobilizing intracellular Ca$^{2+}$. The CaR is found in high concentrations on the surface of parathyroid cells, on calcitonin-secreting C-cells of the thyroid, at various sites along the nephron, and in certain areas of the brain (Coburn et al., 1999).

In different tissues of the same species, the Ca$^{2+}$ receptor has been shown to be identical to that expressed in parathyroid cells (Nemeth, 1996). The human parathyroid CaR possesses an unusually large NH$_2$-terminal extracellular domain with 11 potential glycosylation sites, a membrane spanning domain containing seven membrane-spanning regions, and a relatively large COOH-terminal cytoplasmic domain. The CaR shares limited homology only with metabotropic glutamate receptors (mGluRs) (Nemeth, 1996). Analysis of the sequence of the CaR suggests a likely role of the extracellular domain in binding extracellular Ca$^{2+}$ and activating the receptor. Similarly, mGluRs have the extracellular binding domain for glutamate. Since the CaR is designed to sense plasma levels of Ca$^{2+}$, its affinity for extracellular Ca$^{2+}$ is presumably in the millimolar range. There are regions of the receptor that are enriched with acidic amino acids. Since these
regions are enriched in negative charges, this could play a role in binding extracellular Ca\textsuperscript{2+}.

The CaR is relatively indiscriminate with respect to ions or compounds possessing net positive charges, such as: \( \text{La}^{3+} > \text{Gd}^{3+} > \text{Be}^{2+} > \text{Ca}^{2+} = \text{Ba}^{2+} > \text{Sr}^{2+} > \text{Mg}^{2+} \) (Nemeth, 1996). Likewise, many organic polycations activate the CaR, including simple polyamines (spermine), aminoglycoside antibiotics (neomycin), and polyamino acids (polylysine) or proteins (protamine). All these organic and inorganic polycations mobilize intracellular Ca\textsuperscript{2+} and inhibit hormone secretion in parathyroid cells \textit{in vitro}. They do so, moreover, in the absence of extracellular Ca\textsuperscript{2+}. Various clinical disorders may arise when there is a mutation affecting the CaR, in which the CaR becomes either more or less sensitive to calcium (Coburn, et al., 1999). Mutations in the CaR causing it to be less sensitive to extracellular calcium can lead to familial hypocalciuric hypercalcemia in heterozygotes or to severe infantile hyperparathyroidism in homozygotes. Mutations in the CaR causing it to be more sensitive to extracellular calcium can lead to hereditary forms of hypoparathyroidism.

Calcimimetic drugs, which amplify the sensitivity of the CaR to calcium, can suppress PTH levels, leading to a fall in blood calcium and may be useful therapeutic agents for some diseases of calcium metabolism such as hyperparathyroidism. The first class of compounds that can be considered selective pharmacological probes of the Ca\textsuperscript{2+} receptor are phenylalkylamines typified by NPS R-568 (Nemeth, 1996). A study was done in rats with experimental uremia, to demonstrate the effects of NPS R-568 on a model of secondary hyperparathyroidism, in which there is a proliferation of parathyroid cells. It was found that after 4 days of treatment, the degree of proliferation of the
parathyroid cells was reduced by 50% by high doses of NPS R-568. In another study of uremic rats with osteitis fibrosa, two different doses of NPS R-568 were given daily for 30 days (Coburn, et al., 1999). A dose-related reduction of PTH levels and marked improvement in the degree of osteitis fibrosa were seen.

**CaR in other tissues**

Transcripts of the CaR have been found within the kidney in the following sites: the juxtaglomerular apparatus, along the luminal proximal convoluted tubule, the basolateral surface of the cortical thick ascending limb, and the apical and luminal membrane of the inner medullary collecting duct (Coburn, et al., 1999). The effect of hypercalcemia to lower GFR, to cause increased urinary excretion of Ca$^{2+}$, and to lead to increased volumes of dilute urine may arise through activation of the CaR within various parts of the nephron. Varying levels of CaR expression have also been demonstrated in diverse regions of the brain using immunohistochemistry and in situ hybridization with CaR specific antisera and probes (Yamaguchi, et al., 2000).

**Extracellular Ca$^{2+}$-sensing mechanisms on bone cells**

There are grounds to believe that both osteoblasts and osteoclasts possess some mechanism capable of sensing extracellular Ca$^{2+}$ (Nemeth, 1996). During bone remodeling, osteoclasts and osteoblasts function in local environments characterized by dramatic fluctuations in extracellular Ca$^{2+}$ concentrations (Silver et al., 1988). Increased concentrations of extracellular Ca$^{2+}$ elicit increases in intracellular Ca$^{2+}$ in osteoclasts and alter their morphology, and these effects are associated with an inhibition of bone
resorption in vivo (Coburn, et al., 1988). High concentrations of extracellular Ca\(^{2+}\) (3 - 10 mM) are required to affect the behavior of osteoclasts. Calcimimetic compounds are generally ineffective in altering intracellular Ca\(^{2+}\) in osteoclasts, suggesting that the putative receptor on the osteoclast is structurally distinct from that on parathyroid cells (Coburn, et al., 1999). Fukumoto (1998), showed through functional and histological studies that the calcium-sensing mechanism in osteoclasts is a ryanodine receptor-like molecule in the plasma membrane, while the calcium sensing mechanism in osteoblasts may be similar to the calcium receptor identified in the parathyroid gland.

The nature of the receptor mediating the effects of Ca\(^{2+}\) on osteoblasts is also debated. Increasing the concentration of extracellular Ca\(^{2+}\) above 3mM stimulates DNA synthesis in osteoblast-like MC3T3-E1 cells (Sugimoto, et al., 1993). The proliferative effect of extracellular Ca\(^{2+}\) may be mediated by increased production of insulin-like growth factors (Sugimoto, et al., 1994). The receptor mediating this effect on proliferation appears to be structurally different from the CaR present on parathyroid cells because a variety of osteoblast-cell lines, including MC3T3-E1 cells, do not express mRNA for the CaR and because increased concentrations of extracellular Ca\(^{2+}\) do not evoke corresponding increases in intracellular Ca\(^{2+}\) in osteoblast-like cells (Nemeth, 1996).

On the other hand, some studies suggest that the receptor for Ca\(^{2+}\) on osteoblasts is similar to the CaR. Recently, in the mouse osteoblastic cell line MC3T3-E1, it was shown through both immunocytochemistry and Western blot analysis, using an antiserum specific for the CaR, that CaR protein was detected in these cells (Yamaguchi et al., 2000). Northern analysis also confirmed the presence of CaR transcripts. Exposure of
MC3T3-E1 cells to high Ca$^{2+}$ (up to 4.8 mM) or the polycationic CaR agonists, neomycin and gadolinium, stimulated both chemotaxis and DNA synthesis in MC3T3-E1 cells (Yamaguchi et al., 2000).

Yamaguchi et al. (2000) have recently demonstrated the expression of CaR transcripts and protein in several osteoblastic cell lines, including murine MC3T3-E1 cells, using multiple detection methods. To date however, there has been no rigorous documentation of the CaR’s involvement in the regulation of osteoblast function. Several studies have reported that Ca$^{2+}$-activated K$^+$ channels similar to those present in and activated by the CaR in neurons and lens epithelial cells are also expressed in primary cultures of chick osteoblasts as well as in the human clonal osteoblastic cell lines G292, MG-63, and SaOS-2 (Yamaguchi, et al., 2000). If Ca$^{2+}$-activated K$^+$ channels in osteoblastic cells are regulated by the CaR in a manner similar to those in neurons and lens epithelial cells, such channels could provide a useful parameter for showing that the CaR is not only expressed in osteoblastic cells but is also functionally active. It was found that the Ca$^{2+}$ receptor expressed by MC3T3-E1 cells is functionally coupled to a Ca$^{2+}$-activated K$^+$ channel, as revealed by its activation on raising the level of extracellular calcium or addition of the selective CaR activator, NPS R-467, but not by the less active stereoisomer, NPS S-467 (Yamaguchi, et al., 2000).

In MC3T3-E1 cells, a concentration-dependent increase in chemotaxis was demonstrated between 1.8 and 5 mM of Ca$^{2+}$ (Godwin, et al., 1996). Evidence for receptor-mediated signaling by extracellular calcium was indicated by results obtained using two inhibitors, pertussis toxin and U-73122. Pertussis toxin, an inhibitor of G-protein activation, blocked almost all of the extracellular Ca$^{2+}$-stimulated chemotaxis.
This result suggests that a G-protein linked receptor must be involved in extracellular Ca\textsuperscript{2+}-mediated chemotaxis. Signaling events downstream of G-protein linked receptors include the stimulation of phospholipase C (PLC) activity. In the presence of U-73122, which blocks the activation of PI-PLC, extracellular Ca\textsuperscript{2+}-directed chemotaxis was almost completely inhibited. This result indicates that extracellular Ca\textsuperscript{2+}-mediated chemotaxis of MC3T3-E1 cells requires PLC (Godwin et al., 1996).

**Prostaglandins and tooth movement**

The precursor for prostaglandins (PGs) and leukotrienes is arachidonic acid, which is released from the phospholipids of the cell membrane by the action of phospholipase enzymes. Arachidonic acid is metabolized by two main enzyme pathways: cyclooxygenase and lipoxygenase. Cyclooxygenase activity results in the formation of PGs and thromboxanes. Lipoxygenase activity results in the formation of leukotrienes and HETE’s (Herschman, 1994).

PGs may play an important role in tooth movement. Tooth movement requires both bone resorption and new bone formation, and PGs can stimulate both processes (Pilbeam et al., 2002). PGs produced by osteoblasts are potent stimulators of bone resorption. Many studies indicate that PGs promote the differentiation of osteoclasts from hematopoietic precursors and enhance the effects of other resorption agonists (Okada et al., 2000; Pilbeam et al., 2002). Yamasaki et al. (1980), demonstrated on a rat model that injection of PGs increased osteoclast numbers. Clinical use of PGE\textsubscript{1} has been shown to increase the efficiency of orthodontic tooth movement (Sandy, et al., 1993).

Indomethacin, a cyclooxygenase inhibitor, which inhibits PG synthesis, blocked the
appearance of osteoclasts (Yamasaki, et al., 1980). Chumbley et al. (1986), showed that with indomethacin administration, the rate of tooth movement in the experimental group was approximately one half of the controls in mongrel cats. This reflects the importance of prostaglandins’ role in bone resorption during orthodontic tooth movement.

It has been shown that prostaglandins of the E series are the prostaglandins with greatest activity in bone (Norrdin, et al., 1990). They are found in areas of bone resorption, inflammation, and sites of tooth movement. With pharmacological administration of prostaglandins, bone formation is also seen. In this way, prostaglandins are involved in both bone resorption and bone formation (Norrdin, et al., 1990).

**Cyclooxygenase-2**

Cyclooxygenase (COX) is the major rate-limiting enzyme that converts arachidonic acid released from membranes into prostanoids (Pilbeam et al., 2002). There are two isoenzymes encoded by separate genes for COX, COX-1 and COX-2. COX-1 is constitutively expressed in bone and COX-2 is inducible. Many factors that regulate bone metabolism also regulate the expression of COX-2 in osteoblasts. Acute PG responses in bone to cytokines, growth factors and hormones are dependent on the expression of COX-2.
Hypothesis

We hypothesize that extracellular calcium can induce COX-2 expression and PG production in osteoblasts and that this induction is transcriptionally mediated.

Specific Objectives

**Aim 1.** To examine the effects of increasing extracellular Ca\(^{2+}\) concentration on steady state levels of COX-2 mRNA levels in cultured primary calvarial osteoblasts.

**Aim 2.** To demonstrate that the Ca\(^{2+}\) -induced changes in COX-2 mRNA expression are translated into COX-2 protein expression and PG production.

**Aim 3.** To correlate the changes in COX-2 mRNA expression with luciferase activity using primary osteoblasts from calvariae of mice transgenic for COX-2 promoter--luciferase reporter DNA (Pluc mice).
Materials and Methods

Materials

Murine COX-2 complementary DNA (cDNA) and DNA constructs consisting of −371 to +70 base pairs of the COX-2 promoter fused to a luciferase reporter gene in pXp-2 vector (Pluc) were donated by Harvey Herschman (UCLA, Los Angeles, CA). Transgenic mice carrying Pluc were developed in a CD-1 background at the University of Connecticut Transgenic Animal Facility. Polymerase chain reaction (PCR) was used to amplify cDNA for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) with a control amplifier purchased from Clontech (Palo Alto, CA). Puromycin was purchased from Biomol (Plymouth Meeting, PA). COX-2 antibodies were purchased from Caymen Chemicals (Ann Arbor, MI). All other chemicals were purchased from Sigma (St. Louis, MO).

Primary osteoblastic cells

Primary osteoblastic cells were obtained by sequential digestion of neonatal Pluc mice calvaria. Calvariae were excised, stripped of connective tissue, and washed with phosphate-buffered saline (PBS). Sequential digestions with collagenase P and trypsin were performed to yield 5 populations of cells and populations 2-5 were pooled in 10% FCS-DMEM media and grown to confluence before replating for the experiments. Cells for experiments were plated at 5,000 cells/cm² in 6-well culture dishes in a phenol red-
free DMEM with 10% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (50 microgram/ml) and grown to confluence over 6 days. All cells were cultured at 37 degrees C in a humidified atmosphere of 5% CO₂ in air. On the 7th day, cells were treated with added calcium in the form of CaCl₂ in serum-free DMEM containing 1 mg/ml BSA. CaCl₂ was added in the amount necessary to bring the basal calcium concentration of 1.8 mM in the DMEM up to the required concentration.

*Northern blot analysis*

Total RNA was extracted using Tri-Reagent (MRC, Cincinnati, OH). The manufacturer’s instructions were followed. 10-20 micrograms of total RNA were run on a 1% agarose-2.2 M of formaldehyde gel, transferred to a nylon membrane by capillary pressure and fixed to the membrane by UV radiation. After 3 hours of prehybridization in a 50% formamide solution at 42 degrees C, filters were hybridized overnight in a similar solution in rotating cylinders at the same temperature with a random (³²P) deoxycytosine triphosphate (dCTP)-labeled cDNA probe for COX-2 and later for GAPDH. Filters were washed in a 1x SSC, 1% sodium dodecyl sulfate (SDS) solution at 65 degrees C, and three more times in the latter solution at room temperature. After washing, the filters were exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) at -70 degrees C. Signals were normalized to GADPH. Bands were scanned into the computer and the band density was quantified by the National Institute of Health (NIH) Image 1.61 software.
Western blot analysis

Cells were washed with PBS two times, lysed with SDS buffer (62.5 mM of Tris-HCl, pH 6.8, 2% wt/vol SDS, 10% glycerol, 50 mM of dithiothreitol (DTT), and 0.1% wt/vol bromphenol blue), and scraped into a microcentrifuge tube. The extraction mixture was centrifuged at 14,000 g for 30 minutes. The supernatants were sonicated 10-15 s, heated to 95 degrees C for 5 minutes, cooled on ice, and centrifuged for 5 minutes. Equal amounts of protein (25 microgram), determined by BCA assay, were run on a 10% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane. Membranes were washed with Tris-buffered saline (TBS; pH 7.6), blocked with blocking buffer (1x TBS, 0.1% Tween-20 with 5% wt/vol dry skim milk), and incubated with primary antibody in blocking buffer. After washing with TBS with 0.1% Tween-20 (TBST), the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody and HRP-conjugated antibiotin antibody. After washing with TBST, the signal was detected with LumiGLO chemiluminescent reagent (New England Biolabs).

Medium PGE₂ analysis

PGE₂ levels in the culture medium were measured by radioimmunoassay using antibody purchased from Dr. Lawrence Levine (Brandeis University, Waltham, MA) as previously described (Raisz, 2001). The lower detection limit of the assay for PGE₂ was 50 pg/ml or
0.14 nM. The means and standard error of the mean (SEM) were calculated for n=3 wells in each group.

*Luciferase Assay*

Cells were lysed using a commercially available kit (Promega, WI), and the supernatant assayed for luciferase activity with a Berthold LB 9501/16 luminator (Wallac, Gaithersburg, MD). Luciferase activity, in counts per second, was normalized to total protein in sample by bichoninic acid assay. The mean ± SEM was calculated for n=3 wells for each group, and each experiment was repeated three times.

*Statistical Analysis*

The statistical significance of the differences among the means was determined by analysis of variance (ANOVA), with post-hoc comparison of more than two means by Bonferroni method. The computer program Sigma Stat (Jandel Scientific, San Rafael, CA) was used to run the statistics.
Results

Time course for the induction of COX-2 mRNA by Ca$^{2+}$

Calvarial osteoblastic cultures were treated with 5 mM Ca$^{2+}$, and the expression of COX-2 mRNA determined by Northern blot analysis over a period of 24 h. COX-2 mRNA was not detectable in vehicle-treated cultures (Figure 1). Induction of COX-2 mRNA was seen as early as 30 minutes. COX-2 mRNA (normalized to GAPDH mRNA) peaked at 6-9 h. The COX-2 mRNA level remained elevated at 24 h.

Dose response for the induction of COX-2 mRNA and protein expression by Ca$^{2+}$

Cultures of primary calvarial cells were treated for 6 h with Ca$^{2+}$ at increasing doses and Northern blot analysis was performed (Figure 2). Ca$^{2+}$ levels were raised through addition of CaCl$_2$ as described in Methods and Materials. The basal Ca$^{2+}$ concentration in the culture medium was 1.8 mM. There was a dose-dependent increase in COX-2 mRNA (normalized to GAPDH). COX-2 mRNA expression was induced at 3 mM of Ca$^{2+}$ and peaked at 7.5 mM calcium.

The induction of COX-2 protein was examined by Western blot (Figure 2). The induction of COX-2 protein by increasing Ca$^{2+}$ was studied after 6 h of treatment. There was a dose dependent rise in COX-2 protein levels with administration of Ca$^{2+}$. Induction of COX-2 protein was seen at 3 mM Ca$^{2+}$ and induction peaked at 20 mM Ca$^{2+}$. 
Ca²⁺ induction of PGE₂ production

Cumulative medium PGE₂ production was assayed in cultures of calvarial osteoblasts treated with 5 mM Ca²⁺ for up to 24 h. (Figure 3). In vehicle-treated cultures, levels of PGE₂ were at the lower detection limits of the assay (50 pg/ml) and did not change over the 24 h period. In cultures treated with 5 mM of Ca²⁺, cumulative PGE₂ levels were increased at 3 h and continued to rise over the 24 h period. The steepest rise in cumulative PGE₂ levels was between 3 and 9 h. At 24 h, cells treated with Ca²⁺ had accumulated 9000 pg/ml of PGE₂, approximately 200 times the accumulation in vehicle-treated cells.

Ca²⁺ induction of COX-2 promoter activity in primary osteoblasts

The cells used in the above experiments were derived from mice transgenic for -371/+70 bp of the murine COX-2 promoter fused to a luciferase reporter gene. To determine if the induction of COX-2 gene expression was transcriptionally mediated, similar experiments were performed as above and luciferase activity was measured. Luciferase activity was normalized to total protein in the sample to account for changes in cell number.

The time course for stimulation of luciferase activity in calvarial osteoblasts is shown in Figure 4. Cells were treated with 5 mM Ca²⁺. Ca²⁺ stimulated an 8-fold increase in luciferase activity, with the peak effect occurring at 6 h. The dose response for the Ca²⁺ induction of COX-2 promoter activity in primary osteoblasts is shown in Figure 5. Cells were treated for 6 h. Luciferase activity rose with increasing Ca²⁺ doses of 1.8, 3, 5, and 7.5 mM, peaking at 10 mM calcium.
We examined the regulation of COX-2 and PGE$_2$ production by extracellular Ca$^{2+}$ in primary calvarial osteoblasts. COX-2 mRNA was not detectable in vehicle-treated cultures (1.8 mM Ca$^{2+}$) but was rapidly induced by addition of Ca$^{2+}$ to the medium. The induction of COX-2 mRNA was seen as early as 30 min, peaked at 6-9 h, and mRNA levels continued to be elevated at 24 h. Induction was dose-dependent, beginning at 3 mM and peaking at 10 mM. Ca$^{2+}$ also induced COX-2 protein expression dose-dependently, but the peak effect occurred at 20-40 mM. The difference in peak effects of Ca$^{2+}$ on COX-2 mRNA and protein suggest that the higher concentrations of Ca$^{2+}$ may have effects on COX-2 protein expression. It is possible that high levels of Ca$^{2+}$ could increase translation of COX-2 mRNA into protein or increase the half-life of COX-2 protein by inhibiting enzymes responsible for the degradation of COX-2 protein.

Measurement of cumulative medium PGE$_2$ in calvarial osteoblasts treated with Ca$^{2+}$ (5 mM) showed that Ca$^{2+}$ markedly induced PGE$_2$ production. Despite the constitutive expression of COX-1 in these cells, little PGE$_2$ was produced in vehicle-treated cultures. The rise in PGE$_2$ in Ca$^{2+}$-treated cells correlated with the time course for the Ca$^{2+}$-induction of COX-2 mRNA. Because Ca$^{2+}$ induced COX-2 mRNA and protein expression from undetectable levels, it is not possible to correlate the amplitude of mRNA or protein induction with the amplitude of PGE$_2$ induction. Moreover, since the levels of PGE$_2$ in control cultures were at the lower limit of detection in the assay, the calculation of the Ca$^{2+}$-induced PGE$_2$ increase (about 200-fold) may be an underestimate. Ca$^{2+}$ is the most potent inducer of PGE$_2$ production in these cells by any
agent examined to date (Pilbeam, unpublished observations). It is possible that Ca\(^{2+}\) may also be regulating the activity of phospholipases that release the substrate for COX-2, arachidonic acid, in these cells.

Ca\(^{2+}\) also induced luciferase activity in these primary calvarial cells which carried a transgene consisting of 371 bp of the COX-2 promoter fused to a luciferase reporter. The induction of COX-2 promoter activity paralleled the induction of COX-2 mRNA in both time course and dose response. Hence, the Ca\(^{2+}\)-induction of COX-2 expression appears to be largely transcriptionally mediated. The goal now will be to identify the sites in the COX-2 promoter (and the transacting factors binding to them) which are involved in the transcriptional induction.

Another future goal will be to determine the signaling pathways involved in the Ca\(^{2+}\) induction of COX-2 expression in osteoblasts. Fluid shear stress can also induce COX-2 expression in osteoblasts (Wadhwa et al., 2002). Inhibition of the mitogen activated protein kinase pathway (ERK) could largely prevent this induction. The ERK pathway has previously been shown to be involved in the ability of Ca\(^{2+}\) to stimulate osteoblastic chemotaxis (Godwin, et al., 1997). Hence, the ERK signaling pathway is a likely candidate to be involved in the Ca\(^{2+}\) induction of COX-2 expression.

There is to date much controversy whether or not bone cells express the same extracellular Ca\(^{2+}\) receptor (CaR) as that identified in parathyroid and kidney cells. It is clear that bone cells possess a calcium sensing mechanism, but it cannot be proven that this is the CaR isolated from parathyroid cells at this time. Our results demonstrate that a calcium sensing mechanism does exist on primary calvarial osteoblasts, but whether this mechanism resembles the CaR was not determined through our study.
Since bone remodeling is a key feature during tooth movement, it is therefore of great interest to the orthodontist. Previous studies have shown that extracellular calcium may serve as a coupling agent between bone formation and resorption, by inhibiting osteoclastic activity and stimulating chemotaxis and mitogenesis of osteoblasts (Godwin, et al., 1996). It has been shown that calcium is released locally in levels as high as 40 mM in sites of bone resorption (Silver, et al., 1988). It would be of interest to determine whether the extracellular calcium environment can be regulated locally to enhance or inhibit tooth movement.

On a systemic level, drugs that act on the Ca\(^{2+}\) receptor to alter circulating levels of PTH and calcitonin might provide novel treatments for osteoporosis and for hyperparathyroidism. Calcimimetic and calcilytic compounds could provide new therapeutic options in preventing and treating various bone and mineral disorders. Since this receptor is expressed on many other cell types, drugs acting on Ca\(^{2+}\) receptors could be useful in treating disorders beyond those involving bone and mineral metabolism (Nemeth, 1996).
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Figure 1. Time course of calcium induction of COX-2 mRNA expression in primary calvarial osteoblasts. Cells were treated with 5 mM calcium for times indicated. The housekeeping gene GAPDH is shown to assess loading.
Figure 2. Dose response of calcium induced COX-2 mRNA and protein expression in primary calvarial osteoblasts. (A) Northern blot analysis for COX-2 and GAPDH mRNA. The housekeeping gene GAPDH is shown to assess loading. Cells were treated for 6 hours. (B) Western blot analysis for COX-2 protein. Cells were treated for 6 h.
Figure 3. Calcium induction of PGE₂ production. Cultures of calvarial osteoblasts were treated with 5 mM calcium for up to 24 h. In vehicle-treated cultures levels of PGE₂ were at the lower detection limits of the assay (50 pg/ml). Each symbol represents the mean and SEM of 3 samples. aSignificantly different from respective control, P<0.05, bP<0.01.
Figure 4. **Calcium induction of COX-2 promoter activity in primary osteoblasts: Dose Response.** Luciferase activity was measured in primary osteoblasts from Pluc mice to study transcriptional regulation of the COX-2 promoter. Cells were derived from mice transgenic for -371/+70 bp of the murine COX-2 promoter fused to a luciferase reporter gene. Each symbol represents the mean and SEM of n=6 samples. aSignificantly different relative to control, P<0.01.
Figure 5. Calcium induction of COX-2 promoter activity in primary osteoblasts: Time Course. Cells were treated with 5 mM calcium. All symbols are mean and SEM of n=6 samples. aSignificant difference from respective control, P<0.05; bP<0.01.
References


