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FAST TRACK

Identification of Signal Transduction Pathways and Promoter Sequences That Mediate Parathyroid Hormone 1–38 Inhibition of Osteoprotegerin Gene Expression

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Abstract Osteoprotegerin (OPG), a secreted member of the tumor necrosis receptor superfamily, is a potent inhibitor of osteoclast formation and bone resorption. Parathyroid hormone (PTH), a potent inducer of osteoclast formation, suppresses OPG mRNA expression in vitro and in vivo. To determine the molecular basis of this inhibition, we analyzed the effects of PTH on the human OPG promoter (−5917 to +19) fused with β-galactosidase reporter gene in stable and transient transfections into rat osteoblast-like UMR106 cells. The effect of PTH on OPG promoter expression was biphasic and concentration-dependent. PTH (1–100 nM) induced the transcriptional activity of the OPG promoter (1.7-fold) at 8 h followed by a gradual decrease with maximal inhibition (6.6-fold) at 24–48 h. To ascertain the signal transduction pathways mediating PTH (1–38) effects on OPG gene expression, we compared the effects of PTH with PTH analogs, parathyroid hormone-related protein 1–34 (PTHrP 1–34), forskolin, 3-isobutyl-1-methylxanthine (IBMX), dibutyryl cAMP, phorbol-12-myristate-13-acetate (PMA), thapsigargin and calcium ionophore A23187. PTH 1–31 and PTHrP 1–34, which stimulate the cAMP/PKA pathway, and other activators of cAMP/PKA, forskolin, IBMX, N^6, $O^{2'}$-dibityryl adenosine 3′,5′-cyclic monophosphate (dibutyryl cAMP), all elicited a similar biphasic response on OPG promoter expression. PTH analogs PTH 3–34 and PTH 7–34, that do not stimulate cAMP production, had no effect on OPG expression. In contrast, phorbol-12-myristate-13-acetate (PMA), an activator of PKC, stimulated OPG promoter expression, while thapsigargin and calcium ionophore A23187, which increase intracellular Ca^{2+}, showed a dose-dependent inhibition of OPG promoter expression. To delineate the promoter sequences that mediate the inhibitory effects of PTH on OPG transcription, we analyzed systematic deletions of the OPG promoter for responsiveness in transient transfection assays. The major inhibitory effects of PTH were localized to 391 bp (−372 to +19) of the proximal promoter. Deletions of the promoter region led to a complete loss of responsiveness. Taken together, these results demonstrate that the inhibitory effects of PTH on OPG are mediated at the transcriptional level through *cis* elements in the proximal promoter. The similar biphasic response of OPG to PTH, PTH 1–31, PTHrP 1–34, forskolin, IBMX and dibutyryl cAMP suggests that PTH regulates OPG transcription via activation of the cAMP/PKA signal transduction pathway. J. Cell. Biochem. 84: 1–11, 2002.

Key words: hPTH 1–38; osteoprotegerin (OPG); OPG promoter; gene expression; UMR106 cells

Osteoprotegerin (OPG), a soluble member of the TNF receptor superfamily, inhibits osteoclast formation at a late stage of their development [Simonet et al., 1997; Tsuda et al., 1997; Mizuno et al., 1998; Morinaga et al., 1998; Yasuda et al., 1998a,b; Shalhoub et al., 1999]. In bone, OPG is produced locally and acts as a paracrine factor within the bone micro-environment to decrease bone resorption [Hofbauer and Heufelder, 1997; Hofbauer et al., 2000], and by injection prevents bone loss in rats following ovariectomy [Simonet et al., 1997]. Administration of OPG inhibits osteoclastogenesis and bone resorption response to parathyroid hormone-related protein (PTHrP), 1,25 dihydroxyvitamin D3 (Vit-D), interleukin-1b (IL-b), tumor necrosis factor α (TNFα), low calcium, tumor-induced humoral hypercalcemia, bone

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metastasis, periodontal disease, and adjuvant arthritis [Akatsu et al., 1998; Yamamoto et al., 1998; Kong et al., 1999; Morony et al., 1999; Capparelli et al., 2000; Honore et al., 2000; Min et al., 2000; Teng et al., 2000]. Transgenic mice over-expressing OPG develop osteopetrosis due to the failure of osteoclast formation [Simonet et al., 1997] and targeted deletion of the OPG gene results in severe, early-onset osteopenia [Bucay et al., 1998; Mizuno et al., 1998]. Consistent with its important regulatory role in bone, OPG expression by stromal/osteoblastic cells is altered in response to osteotropic factors, such as 1,25 dihydroxyvitamin D3 [Hofbauer et al., 1998; Horwood et al., 1998], estrogen [Hofbauer et al., 1999a], glucocorticoids [Vidal et al., 1998a], IL-1 [Hofbauer et al., 1998, 1999b; Vidal et al., 1998b], IL-11 [Horwood et al., 1998], TNF [Brandstrom et al., 1998b; Hofbauer et al., 1999b], bone morphogenetic protein-2 [Hofbauer et al., 1998], transforming growth factor β1 [Murakami et al., 1998; Takai et al., 1998], basic fibroblast growth factor [Nakagawa et al., 1999], prostaglandin E2 (PGE_2) [Brandstrom et al., 1998a], and parathyroid hormone (PTH) [Horwood et al., 1998; Murakami et al., 1998; Lee and Lorenzo, 1999], which either enhance or inhibit osteoclast formation. Recently, we demonstrated in vivo that OPG protein is expressed predominantly in pre-osteoblasts, osteoblasts, and bone lining cells, and that OPG mRNA is down-regulated by a single subcutaneous injection or infusion of PTH [Onyia et al., 2000; Ma et al., 2001]. The inhibitory effect of PTH on OPG in vivo had been noted in cultured osteoblasts in vitro [Horwood et al., 1998; Murakami et al., 1998; Lee and Lorenzo, 1999; Onyia et al., 2000]. However, the molecular mechanism by which PTH down-regulates OPG expression to elicit a resorptive effect in bone is not understood.

In the present work we examined how PTH influences OPG gene transcription. We analyzed the human OPG promoter (−5917 to +19) fused with β-galactosidase reporter gene in stably and transiently transfected UMR106 osteoblast-like cells. To ascertain the signal transduction pathways mediating PTH 1–38 effects on OPG gene expression, we compared the effects of PTH with PTH analogs: PTHrP 1–34, forskolin, 3-isobutyl-1-methylxanthine (IBMX), dibutyryl cAMP, phorbol-12-myristate-13-acetate (PMA), thapsigargin, and calcium ionophore A23187. Lastly, we delineated the promoter sequences that mediate the inhibitory effects of PTH on OPG transcription by analyzing systematic deletions of the OPG promoter for responsiveness in transient transfection assays.

MATERIALS AND METHODS

Reagents and Media

Synthetic human PTH 1–38, PTH 1–31, PTHrP 1–34 and bovine PTH 3–34, and 7–34 were obtained from Bachem (Torrance, CA) and were prepared in a vehicle of acidified saline containing 2% heat inactivated serum. Forskolin, IBMX, dibutyryl-cAMP, calcium ionophore A23187 were purchased from Sigma (St Louis, MO). PMA was obtained from Alexis Corp. (San Diego, CA) and thapsigargin was purchased from Calibiochem (San Diego, CA). Forskolin, IBMX, dibutyryl-cAMP, A23187, and thapsigargin were solubilized in dimethyl sulfoxide and further diluted in serum-free media (SFM) to a final concentration of $\leq$0.5%. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). DMEM/F12 (3:1) medium, L-glutamine, HEPES buffer and Geneticin (G418) were obtained from Gibco/BRL Life Technologies (Gaithersburg, MD).

Cloning of the OPG Promoter

Cloning of the 5.9-kb fragment of the human OPG promoter (pOPG5.9βgal), as well as sequential 5′-deletions of the promoter (pOPG3.6βgal, pOPG1.9βgal, pOPG1.5βgal, pOPG0.9βgal pOPG0.48βgal and pOPG0.2β gal), fused with β-galactosidase (β-gal) reporter gene of the pβgal-Basic reporter vector (Clontech, Palo Alto, CA), was performed using standard cloning procedures as previously described [Thirunavukkarasu et al., 2000a]. All constructs were verified by restriction mapping and DNA sequencing, using the dideoxy-chain termination method.

Sequence Analysis

The GCG Wisconsin package (Genetic Computer Group, Inc., Madison, WI) was used to analyze the OPG promoter for the presence of consensus transcriptional binding sites.

Cell Culture, Transfection Assays, and Analyses

UMR106 rat osteoblastic cell line was maintained in DMEM/Ham's F-12 (3:1) (Life

Technologies, Inc.), supplemented with 10% FBS, 20 mM Hepes, and 2 mM glutamine. All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Experiments were initiated when cells were ≈70–80% confluent.

Stable transfection: The OPG promoter construct pOPG5.9βgal (−5917 to +19), was stably transfected into UMR106 cells using Fugene™ 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) as recommended by the manufacturer. A second plasmid pRc/CMV (Invitrogen, San Diego, CA) encoding the neomycin gene was co-transfected for selection. Forty eight hours after transfection in T25 flasks (Corning Inc., Corning, NY) cells were re-seeded (1:10) into T75 flasks (Corning Inc.) and selected in media containing 1 mg/ml G418, for 10 days. Regular media changes were made at 3–4 day intervals. On the tenth day, randomly selected G418 resistant colonies (containing >50 cells) were picked, expanded in fresh media containing 1 mg/ml G418 and assayed for βgal activity. For analysis of gene expression, the selected colonies were plated in 96-well plates (50,000 cells/well) for 24 h and experiments were initiated following serum withdrawal for 12–16 h. Cells were stimulated with various treatments, as described above, for specified times. Cells were then assayed for activity.

Transient transfection: UMR106 cells were grown to 70% confluence in a T-150 flasks (Corning Incorporated) and transfected using the pooled transfection method [Magnuson et al., 1987; Onyia et al., 1995]. In brief, cells were serum-starved overnight (in medium containing 0.1% FBS) and then transfected for ≈5 h with 20 μg DNA (the OPG promoter-βgal constructs or the negative control promoter-less plasmid pβgal-Basic) in Fugene™ 6 reagent (Roche Molecular Biochemicals) as recommended by the manufacturer. The cells were then trypsinized and plated at 50,000 cells per well in 96-well plates in media containing 10% FBS. After allowing 4 h for cells to attach, the medium was replaced with SFM (0.1% FBS) and incubated overnight. Cells were then treated with either vehicle or PTH or other agents at the indicated concentrations and times. After the treatments, cells were lysed in 75 μl of lysis buffer and β-gal activity was assayed in a fixed amount of the extracts (1/3 of the total) using the β-gal reporter gene assay kit (Roche). β-gal enzyme activity was determined and expressed as relative light units or as the percent change over control activity (serum-free controls, with no PTH addition). The results from a representative experiment are shown as the mean ± SEM of 4–12 separate wells.

To obviate pitfalls of using a second or multiple plasmids to determine transfection efficiency [Farr and Roman, 1992], we did not incorporate any internal reference plasmids in our transient transfection experiments. This was done to avoid possible squelching of factors that could arise when co-transfecting multiple plasmids [Farr and Roman, 1992; Selvamurugan et al., 1998]. Differences in transfection efficiency were controlled by the pooled transfection method as described [Magnuson et al., 1987; Onyia et al., 1995]. As a positive control and to verify transfection efficiency, separate plates were transfected with a β-gal expression plasmid (pβgal-Promoter, Clontech), that has the β-gal reporter gene coding region under the control of the SV40 early promoter. β-gal expression was quantified either by histochemical staining or by β-gal enzymatic assay. The transfection efficiency was 85–90% and comparable across plates. Additionally, all of our experiments were repeated multiple times (∼2–6) using multiple clones of the same construct and different preparations of the plasmids. The results obtained under these conditions were similar or identical in nature. Furthermore, the luciferase reporter vector was not used to normalize for transfection efficiency because of our recent finding that cryptic enhancer elements in the luciferase reporter vector pGL3-Basic could mediate transactivation by Cbfal and ERα, leading to spurious background luciferase expression [Thirunavukkarasu et al., 2000b].

β-Galactosidase Assay: Cell extracts were assayed for β-gal activity using the βGal Reporter Gene Assay Kit (Roche Molecular Biochemicals) as recommended by the manufacturer. Assays were done in white, opaque Microlite™ 2+ 96-well plates (Dynex, Chantilly, VA). Luminescence was measured in a MLX microtiter plate luminometer (Dynex) and light integration measured at 2 sec (RLU summed). Results were analyzed using Student's *t*-test, and probabilites (*P*) values of <0.05 were considered significant.

RESULTS

Time and Dose-Dependent Effects of PTH or OPG Promoter Activity

We studied the effects of PTH on OPG transcription by analyzing the expression of a 5.9 kb human OPG promoter fragment (Fig. 1) fused to the β-galactosidase reporter gene in stably transfected UMR106 cells. UMR106 cells were chosen for these studies because of their clonal stability and their receptiveness of transfection. Cells from three randomly picked stable clones were treated with PTH 1-38 at indicated concentrations (10^{-7}–10^{-13} M) for various time intervals (8, 12, 24, and 48 h). OPG promoter expression was determined by assaying the activity of the reporter gene β-galactosidase. Treatment of these clones with PTH 1–38 led to a biphasic and concentration-dependent response. As illustrated in a representative clone (Fig. 2), at 8 h PTH 1–38 (10^{-7}–10^{-9} M) stimulated OPG promoter expression up to 1.7-fold above control levels ($P < 0.001$). This initial increase was followed by a return to basal levels at 12 h and a strong inhibition at 24 and 48 h, with maximal inhibition of 6.6-fold at 48 h (10^{-7} M). Since similar results were obtained in all three randomly chosen stable clones, we selected one for subsequent investigation, and major findings were confirmed in all three clones.

Effects of PTH Analogs on OPG Promoter Activity

Distinct biological activities have been demonstrated for various domains of the PTH molecule that are indicative of specific intracellular signals. Like the parent PTH molecule (1–84), PTH 1–38 has full biological activity and activates both the cAMP/PKA and PLC/PKC pathways [Fujimori et al., 1991, 1992; Jouishomme et al., 1992, 1994; Rixon et al., 1994]. To evaluate the signal transduction mechanisms involved in PTH regulated expression, we analyzed the effect of signal selective analogs of PTH. Specifically, we compared the effects of PTH 1–38 and PTHrP 1–34 (with full biological activity) to PTH 1–31 (which activates the cAMP/PKA pathway) and PTH 3–34 and PTH 7–34 (analogs that do not stimulate the cAMP/PKA pathway). Cells were treated with various analogs of PTH (10^{-8} M) and assayed for promoter response at three time points (8, 24, and 48 h). As shown in Figure 3, PTH 1-31 stimulated OPG promoter activity at 8 h and inhibited OPG promoter activity at 24 and 48 h in the same manner as PTH 1–38 and PTHrP 1–34, while PTH 3–34 and PTH 7–34 failed to regulate any promoter activity. This is indicative of a role for the cAMP/PKA pathway in the biphasic effect of PTH on OPG transcription.

Effects of Forskolin, IBMX, and Dibutyryl-cAMP on OPG Promoter Activity

To directly confirm the involvement of the cAMP/PKA pathway on OPG promoter activation, we used three compounds that increase intracellular cAMP: forskolin, a potent activator of adenylate cyclase; IBMX, a phosphodiesterase inhibitor; and dibutyryl-cAMP, a cell permeable analog of cAMP. Cells were treated with indicated concentrations of forskolin (10^{-4}–10^{-10} M), IBMX (10^{-3}–5×10^{-7} M), and dibutyryl-cAMP (10^{-4}–10^{-7} M), for three time intervals (8, 24, and 48 h). Treatment with

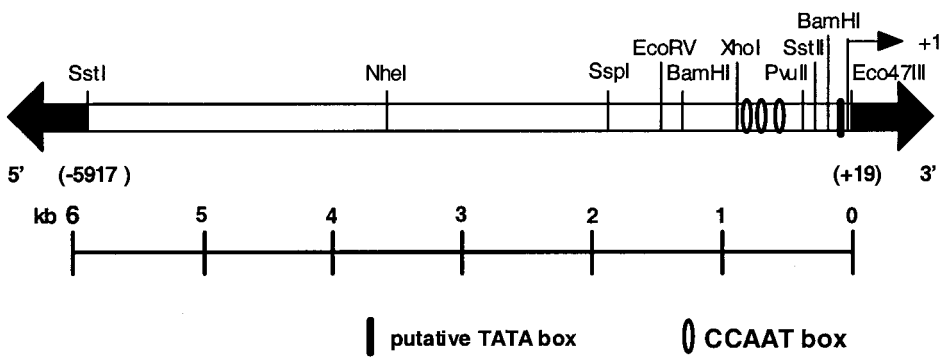


Fig. 1. Schematic representation of the 5.9-kb OPG promoter showing the location of consensus basal elements (TATA and CCAAT boxes) and restriction sites. The arrow represents the transcriptional start site [Morinaga et al., 1998].

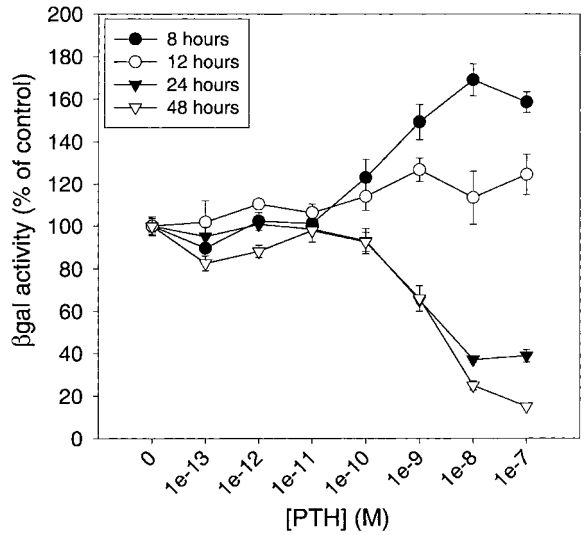


Fig. 2. Effects of PTH-38 on OPG promoter expression in a representative stable clone of UMR106. UMR106 cells stably transfected with pOPG5.9βgal were serum-starved and treated with increasing concentrations of PTH 1–38 for 8, 12, 24, and 48 h. β-gal activity of the cell extract was determined and expressed as the percent change in activity over control activity (serum-free control without PTH 1–38).

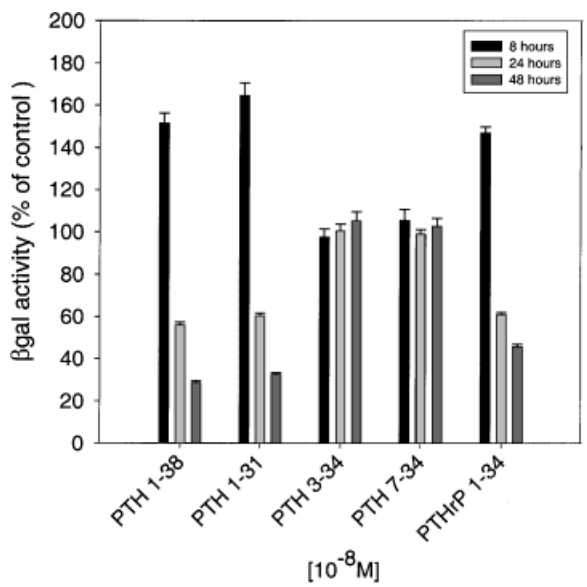


Fig. 3. Effects of PTH analogs and PTHrP 1–34 on OPG promoter expression. UMR106 cells stably transfected with pOPG5.9βgal were serum-starved overnight and treated with 10nM PTH 1-38, PTH 1–31, PTH 3–34 PTH7–34 and PTHrP 1-34, for 8, 24, and 48 h. β-gal activity was determined and expressed as the percent change in activity over control activity (serum-free control without treatment).

forskolin (Fig. 4A) resulted in a dose-dependent stimulation of OPG promoter activity at 8 h with maximal two-fold effect at 10^{-4}–10^{-5}M. At 24 and 48 h, there was a dose-dependent inhibition of OPG promoter activity with a maximal 4.6-fold inhibition at 48 h (10^{-4} M). IBMX treatment (Fig. 4B), which inhibits the degradation of cAMP, resulted in a dose-dependent stimulation of OPG promoter activity at 8 h (2.4-fold at 10^{-3} M), but inhibited promoter expression at 24 and 48 h (inhibition at 2.8-fold at 10^{-3} M). Similarly, dibutyryl-cAMP (Fig. 4C) had a stimulatory effect on OPG at 8 h (2.1-fold), but promoter expression decreased to control levels at 24 h and 3.3-fold below control at 48 h. The stimulation and inhibition of OPG promoter activity by dibutyryl-cAMP were dose-dependent. Therefore, conditions that enhance cAMP accumulation elicit a similar biphasic effect on OPG transcription. Taken together, these results suggest the effects of PTH on the OPG promoter are mediated via the cAMP/PKA signal transduction pathway.

Effects of PMA, Calcium Ionophore A23187 and Thapsigargin on OPG Promoter Expression

Although the results shown in Figs. 3 and 4 suggest that PTH responsiveness depends on the cAMP pathway, they do not exclude a role for the PLC/PKC or Ca^{2+} signaling pathways, which also mediate PTHs effects in the osteoblasts. To explore the role of the PLC/PKC and Ca^{2+} pathways on OPG transcription, we next examined the effect of PMA, a potent activator of PKC, calcium ionophore A23187, which increases intracellular Ca^{2+} and thapsigargin, which increases intracellular Ca^{2+} by inhibiting Ca^{2+} ATPase in the endoplasmic reticular. Cells were treated with indicated concentrations of PMA (10^{-5}–10^{-8} M), A23187 (10^{-7}–10^{-9} M), and thapsigargin (10^{-6}–10^{-9} M) for 8, 24 or 48 h. As shown in Figure 5, at 8 h low doses of PMA (10^{-4}–10^{-7} M) slightly inhibited OPG expression, while higher doses (10^{-5} and 10^{-6} M) stimulated OPG expression 1.4-fold. At 24 and 48 h, PMA treatment stimulated the OPG promoter at all concentrations. A 2.3-fold stimulation was observed at 48 h with 10^{-5}M PMA. In contrast to PMA, treatment with A23187 resulted in a dramatic inhibition of OPG promoter activity at all time points examined (Fig. 6A). A 6.8-fold decrease in OPG promoter expression was observed with 10^{-7}M A23187 [at this concentration of A23187 we did

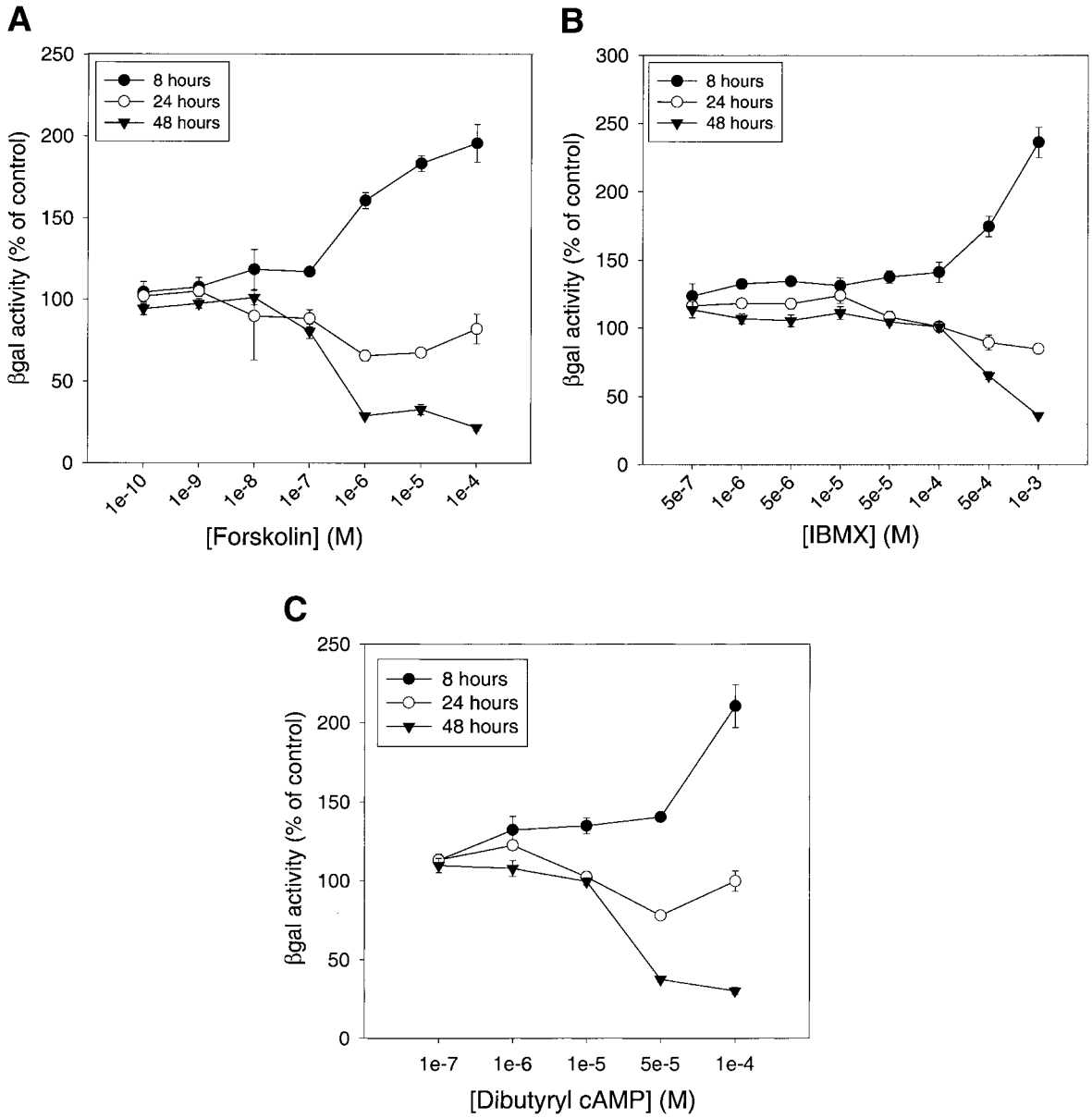


Fig. 4. Effects of compounds that increase cAMP accumulation on OPG promoter expression. UMR106 cells stably transfected with pOPG5.9βgal were serum starved overnight and treated with increasing doses of forskolin (**A**), IBMX (**B**) or dibutyryl cAMP (**C**) for 8, 24, and 48 h. β-gal activity was determined and expressed as the percent change in activity over control activity (serum-free control without treatments).

not detect any toxic effects on the cells (data not shown)]. Also, thapsigargin resulted in a dose-dependent inhibition on OPG promoter activity at 8, 24 and 48 h, to almost four-fold (10^{-7} M) (Fig. 6B, data not shown).

Identification of the PTH Responsive Region of the OPG Promoter

In order to determine the region(s) of the OPG promoter that contributes to PTH 1–38 responsiveness, we made and analyzed systematic 5′-deletions of the promoter (Fig. 7A). The full length and deletion constructs (5.9, 3.6, 1.9, 1.5, 0.9, 0.4, and 0.2 kb OPG-β gal constructs) were transiently transfected into UMR106 cells and analyzed at 24 h for basal expression and PTH 1–38 responsiveness. As shown in Fig. 7B, analysis of β-gal activity demonstrated that sequential deletion of the promoter, up to the 0.4 kb region resulted in an increase in baseline expression as compared to the 5.9 kb promoter. However, deletion of 183 bp region between 0.4

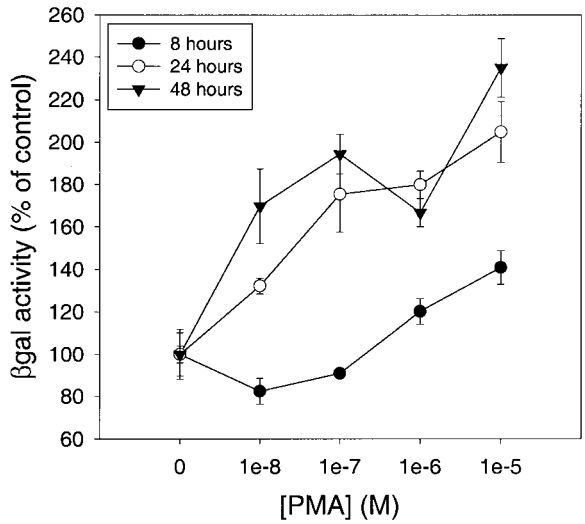


Fig. 5. Effects of PKC activator, PMA, on OPG promoter expression. After overnight serum withdrawal, UMR106 cells stably transfected with pOPG5.9βgal were treated with increasing concentrations of PMA, for 8, 24, and 48 h. β-gal activity was determined and expressed as the percent change in activity over control activity (serum-free control with no PMA).

and 0.2 kb (−372 to −190) resulted in a large drop in promoter activity, and the removal of the proximal promoter (−189 to +19) resulted in an almost complete loss of activity. To simplify our mapping of the PTH response region, we focused only on the profound inhibitory effects of PTH on the OPG promoter. As demonstrated in Figure 7C, sequential removal of the distal promoter (−5936 to −373) had no effect on the PTH inhibitory response of the OPG promoter. Deletion of the proximal 391 bp promoter (−372 to +19) abolished the PTH repressive effect. In this proximal promoter, deletion of the 183 bp region (−372 to −190) resulted in a 50% decrease in PTH repression, while further deletion of the remaining promoter region (−189 to −19) resulted in the complete loss of responsiveness. These results demonstrate that the proximal 391 bp OPG promoter (−372 to +19) is required for both basal expression and the inhibitory effect of PTH.

DISCUSSION

The discovery of OPG as a powerful inhibitor of osteoclast formation has generated great interest in its regulation by physiological agents that regulate bone resorption. We showed in vivo [Onyia et al., 2000; Ma et al., 2001], and there is ample evidence in vitro [Horwood et al., 1998; Murakami et al., 1998; Lee and Lorenzo, 1999; Onyia et al., 2000], that a decrease in OPG expression may be an important factor in explaining PTH stimulation of osteoclast formation.

To elucidate the molecular basis of PTH inhibition of OPG mRNA expression, in the present study we investigated the ability of PTH

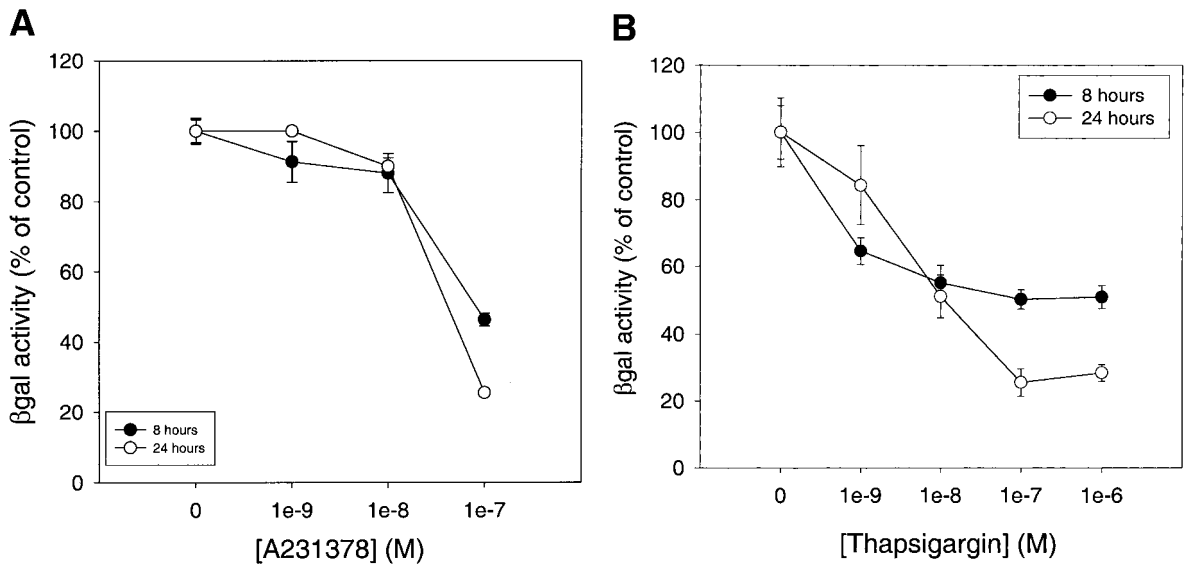
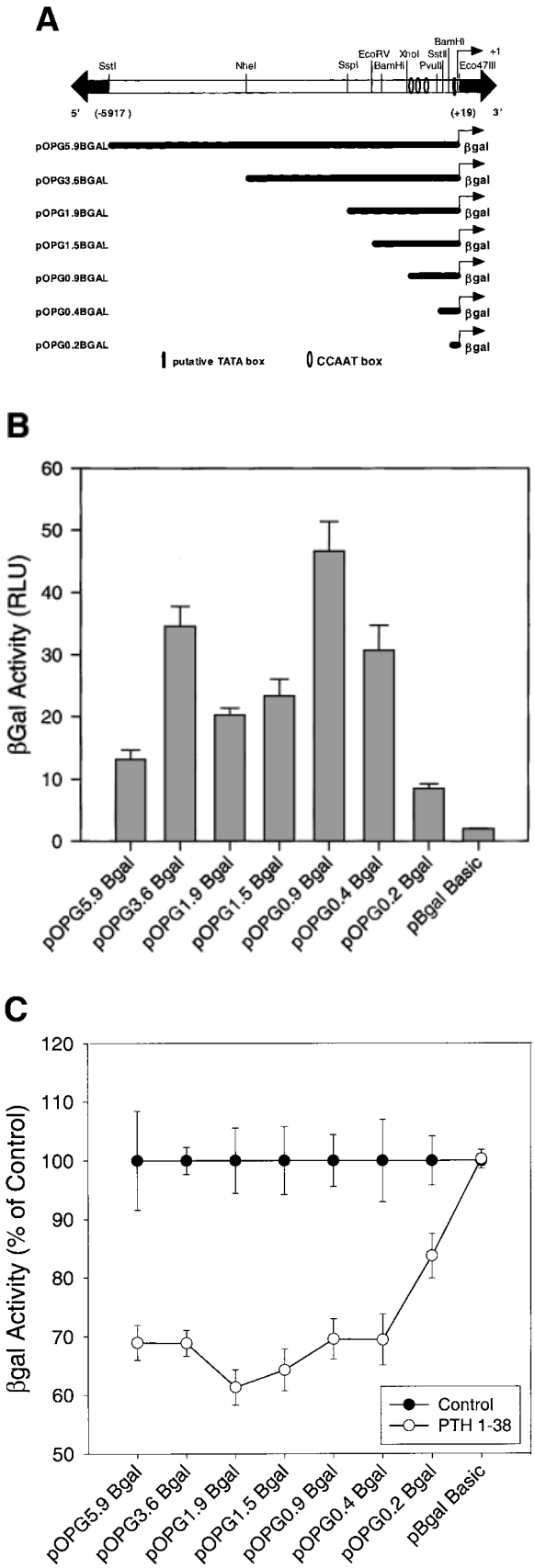


Fig. 6. Effects of compounds that increase intracellular Ca^{2+} on OPG promoter expression. UMR106 cells stably transfected with pOPG5.9βgal were serum-starved overnight and treated with increasing doses of calcium ionophore A23187 (**A**) or thapsigargin (**B**) for 8 and 24 h. β-gal activity was determined and expressed as the percent change in activity over control activity (serum-free control with no treatment).



and signal transduction selective analogs or agents to regulate OPG transcription from the OPG promoter (−5917 to +19). Analyses of OPG promoter linked to β-gal in transient and stable transfection assays showed that PTH inhibited OPG transcription (3–6-fold) in a concentration- and time-dependent manner. Results of our time course studies showed that the inhibitory effects of PTH (10^{-7} to 10^{-10} M) on OPG transcription observed in 24 to 48 h was preceded by an initial small and transient stimulation at 8 h (1.7-fold), with a return to control values by 12 h. This is a consistent effect and confirms at a transcriptional level the result of Lee and Lorenzo [1999] showing a similar biphasic effect of PTH on OPG mRNA in bone marrow cultures. The physiological relevance of this small transient increase in OPG to PTH action is not clear, but should be evaluated in light of the RANKL/OPG ratio, which ultimately determines the ability of osteoblast cells to support osteoclast formation. In studies by Lee and Lorenzo [1999], the initial small increase in OPG mRNA is preceded by a large increase in RANKL mRNA, thus favoring increased osteoclast formation. Similarly, Horwood et al. [1998] have demonstrated that the balance between the levels of RANKL mRNA in calvarial osteoblasts is altered by PTH and is essential for supporting osteoclast formation.

Although PTH activates both the cAMP-PKA and phospholipase C-protein kinase C (PLC-PKC) pathways, previous studies have demonstrated that the cAMP-PKA pathway is required for PTH stimulation of bone resorption [Juppner et al., 1991; Kaji et al., 1992; Ljunggren and Ljunghall, 1993; Greenfield et al., 1995]. Our recent in vivo evidence that PTH inhibits OPG mRNA via the activation of the cAMP-PKA pathway [Onyia et al., 2000], is consistent with the present results in showing that the inhibition of OPG by PTH is transcriptional and mediated primarily by the cAMP/

Fig. 7. Identification of sequences required for the inhibitory effect of PTH on OPG promoter. **A**: Schematic representation of OPG promoter 5′-deletion constructs fused to β-gal reporter gene that were used in transient transfection analysis. **B**: Basal promoter activity of OPG promoter deletion constructs in transient transfection expressed as relative light units (RLU). **C**: The effect of PTH 1–38 on OPG promoter deletion constructs. UMR106 cells were transiently transfected with OPG promoter deletion constructs and treated with PTH 1–38 (5×10^{-7} M) for 24 h. Cell extracts were assayed for β-gal activity and results are expressed as the percent change in activity over control activity (serum-free control with no PTH).

PKA pathway. This conclusion is supported by results demonstrating that PTH 1–31 activates only cAMP-PKA with no demonstrable effects on PLC-PKC [Jouishomme et al., 1994; Rixon et al., 1994]. Additionally, other activators of the cAMP-PKA pathway, forskolin, IBMX, and a cell permeable cAMP analog, dibutyryl cAMP, all elicited a biphasic response similar to that obtained with PTH 1–38. Furthermore, N-terminally truncated PTH analogs, PTH 3–34 and PTH 7–34, that do not stimulate cAMP production [Fujimori et al., 1991, 1992; Jouishomme et al., 1992], had no effect on OPG expression. In contrast, PMA, a direct activator of PKC, stimulated OPG promoter expression, while thapsigargin and calcium ionophore A23187, which increase intracellular Ca^{2+}, showed only a dose dependent inhibition of OPG promoter expression at all time points examined. Taken together, these suggest that multiple signaling pathways can regulate OPG transcription in osteoblasts and could contribute to the modulation of osteoclast formation and bone resorption.

The proximal 1-kb flanking region of the OPG gene has been previously defined and has been shown to contain at least two promoters (one proximal and one distal promoter) defined by three transcription initiation sites (TIS) [Morinaga et al., 1998]. The distal promoter is marked by two weak TIS present 0.6-kb upstream (−646 and −667) from the ATG and direct the expression of a 3-kb transcript in tissues such as brain, placenta, spleen, and prostate [Morinaga et al., 1998; Yasuda et al., 1998a]. The proximal promoter is marked by a stronger TIS at −67 upstream of the initiation ATG and directs the expression of a 2.4-kb mRNA transcript in many tissues [Morinaga et al., 1998]. In previous studies we and other authors have demonstrated that the 2.4-kb transcript (derived from the proximal promoter) is the transcript expressed in bone and that it is inhibited by PTH [Onyia et al., 2000]. To directly delineate regions of the OPG promoter important for responsiveness to PTH we analyzed progressive deletions of the promoter in transient transfection assays. For convenience we focused on the profound inhibitory effects of PTH observed in 24–48 h. Our findings localized the PTH response DNA element of OPG to 0.4 kb (−372 to +19) of the proximal promoter. The proximal promoter region was sufficient for directing both basal expression and the full inhibitory effect of PTH. Together, these results demonstrate that the inhibitory effects of PTH on the OPG gene are mediated at a transcriptional level through DNA elements in the proximal promoter. Computer analysis of the OPG promoter proximal region (−372 to +19) revealed the presence of consensus binding sites for a variety of transcription factors including OSF-2 and AP1 that have been implicated in PTH stimulation of collagenase-3 gene [Porte et al., 1999; Selvamurugan et al., 2000]. Future studies are needed to delineate the exact element(s) through which PTH inhibits OPG and would enhance the understanding of PTH-inhibition of OPG expression to stimulate bone resorption.

In summary, our results provide direct evidence that PTH inhibits OPG expression, at least in part, at a transcriptional level through the activation of cAMP signal transduction. Since the major function of OPG is to inhibit osteoclast formation, we conclude that the decrease in OPG expression is an important event in PTH stimulation of bone resorption.

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