Osteoprotegerin Expression and Secretion Are Regulated by Calcium Influx through the L-Type Voltage-Sensitive Calcium Channel

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Our previous studies showed that 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] modulates the activity of the Caᵥ₁.₂ α-subunit of the L-type voltage-sensitive calcium channel (VSCC) by two temporally distinct mechanisms. First, 1,25(OH)₂D₃ rapidly modulates local Ca²⁺ permeability in the plasma membrane of the proliferating osteoblast. Second, treatment with 1,25(OH)₂D₃ reduces biosynthesis of Caᵥ₁.₂ such that transcript levels are half of original levels after 24 h. Osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) provide important regulatory mechanisms for controlling osteoclastogenesis and Ca⁺² homeostasis. Because they often control Ca²⁺-activated secretion, we investigated the possibility that L-type VSCCs might regulate basal OPG and RANKL secretion in osteoblasts. We also studied 1,25(OH)₂D₃ effects on OPG and RANKL expression. To address this, we measured changes in expression and secretion of OPG and RANKL in MC3T3-E1 cells and calvarial organ cultures after treatment with 1,25(OH)₂D₃, VSCC inhibitors, and inhibitors of Ca²⁺-regulated signaling. RANKL production was increased in calvarial cultures by 1,25(OH)₂D₃ but was essentially undetectable in the medium of MC3T3-E1 cells. In contrast, OPG secretion in both systems was significantly reduced after 24 h treatment with 1,25(OH)₂D₃ by inhibitors of L-type VSCCs and calmodulin-sensitive protein kinases but not by inhibitors of protein kinase A, MAPKs, or other families of VSCCs. OPG secretion was abrogated by transfection with decay cAMP response element binding sites. Our results suggest that OPG secretion is regulated through calmodulin-sensitive protein kinase signaling that depends on the activity of the L-type VSCC and is mediated through the cAMP response element-binding protein. (Endocrinology 145: 426–436, 2004)

THE RATES OF bone formation and resorption are tightly regulated by the activity of osteoclasts and by cells of the osteoblastic lineage (1). Regulation of bone resorption occurs through the osteoblast and involves two key regulators: osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL). OPG is a 380-amino acid secreted protein that lacks membrane and cytoplasmic domains (2). Overexpression of OPG in mice results in osteopetrosis and diminished bone remodeling (2). OPG knockout mice develop osteoporosis and have decreased bone mass, an absence of trabecular structure in their long bones, and poorly developed growth plates (3–5). RANKL is a 317-amino acid peptide and is a binding partner of OPG (6). Two distinct forms of RANKL have been identified, a 45-kDa membrane-associated form (mRANKL) and a 32-kDa soluble form (sRANKL) derived from proteolytic cleavage of the membrane-associated form (4). RANKL knockout mice display severe osteopetrosis (7, 8), whereas administration of RANKL to normal mice results in a rapid onset of hypercalcemia (4). OPG is a soluble receptor that inhibits osteoclastogenesis by binding and neutralizing RANKL.

Resorption is regulated through OPG and RANKL expression by osteoblastic cells and is altered by various osteotropic factors that alter plasma membrane permeability to Ca²⁺, including 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] or ionophores (9–11). 1,25(OH)₂D₃ activates rapid, plasma membrane-initiated signaling events and longer-term nuclear receptor-mediated pathways in osteoblastic cells by different receptor pathways (12). 1,25(OH)₂D₃ activation of the plasma membrane signaling system changes the functional properties of voltage-sensitive Ca²⁺ channels (VSCCs) and alters the expression and activity of protein kinases (13–16). Application of 1,25(OH)₂D₃ increases plasma membrane permeability to Ca²⁺ within milliseconds by shifting the threshold of activation toward the resting potential and increasing the mean open time of the L-type VSCC (16, 17). In the absence of a depolarizing signal, such as would occur on application of a calcitropic peptide hormone, this results only in a local increase in Ca²⁺ and does not translate into a wholesale increase in cytoplasmic Ca²⁺ concentration (17). Previous studies in our laboratory showed that the L-type VSCC, Caᵥ₁.₂, is the primary site for Ca²⁺ influx into the proliferating osteoblast (16). Spontaneous and hormonally regulated opening of Ca²⁺ channels leads to localized elevations of intracellular Ca²⁺ that directly control the release of secretory vesicles (18, 19). A secretory state is thus maintained by the shift to a cellular condition in which Ca²⁺ rapidly cycles across the partially depolarized plasma membrane without a wholesale increase in cellular Ca²⁺ that could have detrimental effects on cell viability (20, 21).
Ca\(^{2+}\) influx also directly or indirectly influences the expression and activity of intracellular protein kinases, including protein kinase A (PKA), Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMK), and MAPK (22–26). PKA, CaMK, and MAPK are protein kinases that play roles in intracellular Ca\(^{2+}\) homeostasis and can potentially phosphorylate the pore-forming Ca\(_{V1.2}\) Subunit of the L-type VSCC (22–24), creating feedback mechanisms for alteration of channel function. In addition to modulating VSCC activity, these signaling molecules can alter gene expression through the function of the cAMP response element (CRE) (27–29). The CRE transcription factor binding site is composed of a consensus palindromic sequence of eight base pairs, TGACGTCA (29), with which CRE-binding protein (CREB) can complex to alter gene transcription. Phosphorylation of serine-133 on CREB by CaMK and CREB. Based on our observations reported here, a model of OPG production and secretion is proposed in which Ca\(^{2+}\) influx into the osteoblast through the L-type VSCC maintains basal OPG secretion. In the presence of 1,25(OH)\(_2\)D\(_3\), we propose that OPG production is attenuated because VSCC maintains basal OPG secretion. In the presence of 1,25(OH)\(_2\)D\(_3\)-treated cultures, and 0.01% (vol/vol) ethanol (EtOH) for 1,25(OH)\(_2\)D\(_3\)-treated cultures, and 0.01% (vol/vol) dimethylsulfoxide (DMSO) for cultures to compare results in a physiologically relevant system that regulates both OPG and RANKL expression and secretion. Because of its ability to affect VSCC activity at two levels, a rapid change in the gating properties followed by a transcriptional down-regulation of L-type channel biosynthesis, we also investigated the potential interactions between 1,25(OH)\(_2\)D\(_3\) and VSCC-regulated Ca\(^{2+}\)-dependent signaling and transcription, specifically that mediated by CaMK and CREB. Based on our observations reported here, a model of OPG production and secretion is proposed in which Ca\(^{2+}\) influx into the osteoblast through the L-type VSCC maintains basal OPG secretion. In the presence of 1,25(OH)\(_2\)D\(_3\), we propose that OPG production is attenuated first through a negative feedback signaling mechanism that reduces plasma membrane Ca\(^{2+}\) permeability and second, through a longer-term down-regulation of VSCC biosynthesis.

### Materials and Methods

**Cell and organ cultures**

MC3T3-E1 cells, a gift from Dr. Renny Franceschi (University of Michigan, Ann Arbor, MI), were plated at 5000 cells/cm\(^2\) and maintained in α-modified Eagle’s media (αMEM) containing ribonucleosides and deoxyribonucleosides, supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES buffer. All culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA). Cultures were maintained in a 37 C humidified chamber with 5% CO\(_2\). The medium was changed every 3 d, and the cell lines were passaged at 80% confluency using trypsin-EDTA. For experimental treatment, cells were plated in 24-well cell culture dishes (Corning Inc., Corning, NY) and allowed to grow for 48 h in serum-containing medium. The cells were then rinsed twice with PBS and then fed with serum-free αMEM supplemented with penicillin, streptomycin, and HEPES. After 24 h incubation in serum-free media, the cells were rinsed with PBS and fresh serum-free media was added that contained the treatment or the appropriate vehicle control: 0.01% (vol/vol) ethanol (EtOH) for 1,25(OH)\(_2\)D\(_3\)-treated cultures, and 0.01% (vol/vol) dimethylsulfoxide (DMSO) for cultures treated with inhibitors to VSCCs, CaMK, MAPK, and PKA.

Timed pregnant outbred female Swiss-Webster mice were obtained from Taconic (Taconic, NY). Calvaria were excised from 4- to 5-old neonates using aseptic technique. Excess soft tissue was removed and the calvaria were cut along the sagittal suture line to produce two hemicalvariae per animal. The calvarial pieces were floated on discs of filter paper in serum-free αMEM as previously described (35). All animals were maintained and used in accordance to the principles set forth in the Guide for the Care and Use of Laboratory Animals and under Institutional Animal Care and Use Committee approved Animal Use Protocol number 1109–2003–0.

Inhibitors and 1,25(OH)\(_2\)D\(_3\) were added to MC3T3-E1 cells after 12 h of serum starvation and directly to calvaria cultures. All inhibitors were added to the cultures for 30 min. The media were aspirated and fresh media containing inhibitors were added. VSCC inhibitors (Alomone Labs, Jerusalem, Israel) were added in the following concentrations: L-type inhibitor, nifedipine (1 µM); T-type inhibitor, sTFX-3.3 (200 nM); P/Q-type inhibitor, ω-agatoxin IVA (1 µM); N-type inhibitor, ω-conotoxin GVIA (1 µM); and R-type inhibitor, SNX-482 (100 nM). Inhibitors for CaMK, PKA, and MAPK pathways (Calbiochem, San Diego, CA) were applied in the same manner as the VSCC inhibitors, in the following concentrations: CaMK inhibitor, KN93 (10 µM); PKA inhibitor, H89 (10 µM); and MAPK pathway inhibitor, PD98059 (10 µM).

### RNA isolation and RT-PCR

Total RNA was extracted from MC3T3-E1 cultures at 80% confluence using the RNeasy kit (Qiagen) and reverse-transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI). cDNA was amplified in 96-well plates in triplicate using primers described in Table 2. Expression of target genes was verified using GAPDH transcripts as a control. 

**RT-PCR**

Expression of Ca\(_{V1.2}\) mRNA was detected using the QuantiTect SYBR Green PCR kit (Qiagen). The primer sequences used were designed based on published species-specific sequences: OPG (accession no. U94331) F-5’, tcctggacactaaacagga and R-5’, acgtgctgctaatcctct (125-bp product); RANKL (AF053713) F-5’-ctggaagaggtagggccagac and R-5’-gagggttgcaacctga (128 bp); Ca\(_{V1.2}\) (Cacna1c) F-5’-gcgtgctgtctgctaggaag and R-5’-gctctgccagggagaata (141 bp); and L19 (M62952) F-5’-cgagtcctgaaagaggtga and R-5’-tcagctgtggctgtctgctg (110 bp). Standards were generated by cloning the PCR product into the pCR2.1 vector using the TOPO T/A cloning kit (Invitrogen). Isolated plasmid was linearized using EcoRV, quantitated, and diluted to use as standards in real-time RT-PCRs. Real-time RT-PCR was performed in the iCycler iQ real-time PCR detection system from Bio-Rad Laboratories, Inc. (Hercules, CA). After 10 min incubation at 95 C, the cycling conditions were as follows: denaturation at 94 C for 45 sec, annealing at 58 C for 45 sec, and extension for 40 sec at 72 C for 45 cycles. Data were analyzed on Prism 3.0 from GraphPad Prism (San Diego, CA).

### Confocal microscopy

Detection of Ca\(_{V1.2}\) subunit was performed using an affinity-purified rabbit polyclonal antibody against Ca\(_{V1.2}\) obtained from Alomone Labs. MC3T3-E1 cells were cultured as described above. Five thousand cells per well were plated in 8-well Lab-Tek chamber-slides (Nalge Nunc International, Naperville, IL). The cells were grown overnight in a 37 C incubator containing 5% CO\(_2\). After 24 h, the cells were washed three times with PBS and then fixed in 4% (vol/vol) paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1% (vol/vol) PBS on ice. After fixation, the cells were rinsed with 0.1% (wt/vol) sodium-azide in PBS for 5 min on ice. To permeabilize and block nonspecific binding, the cells were incubated in blocking buffer containing 5% (vol/vol) normal donkey serum, 0.3% (vol/vol) Triton X-100, and PBS for 30 min at room temperature. The primary antibody was diluted 1:50 with 0.1% (wt/vol) BSA and 0.01% (wt/vol) sodium-azide in PBS, applied to the fixed cells, and allowed to incubate for 1 h at room temperature. After washing three times for 10 min each with 1% (vol/vol) normal donkey serum and PBS, the cells were incubated in the dark for 1 h at room temperature with fluorescein isothiocyanate (FITC) conjugated donkey antirabbit IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:100. The cells were counterstained for 10 min in the dark with the nuclear dye ToPro3 (Molecular Probes, Eugene, OR) diluted 1:4000 in PBS. The cells were washed three times.
for 5 min each in PBS and stored at 4°C until use. The fluorescence was analyzed with an inverted microscope linked to a confocal scanning unit (Axiovert LSM 410; Carl Zeiss, Oberkochen, Germany). To determine specificity of staining, images were compared with cells that were incubated with non-conjugated donkey antirabbit IgG in the absence of primary antibody. Stained cells were compared with samples that were stained with primary antibody that had been preincubated for 1 h with 1 μg of antigenic peptide per microgram of primary antibody.

**Protein measurement of OPG/RANKL**

Cell culture supernatants were collected at the indicated time points and supplemented with 1 μl/ml of protease inhibitor cocktail III (Sigma, St. Louis, MO). The samples were stored at 4°C until use, typically less than 24 h. To measure membrane-associated RANKL after 1,25(OH)2D3 and vehicle treatment, MC3T3-E1 cells were lysed in an extraction buffer containing 8 μM urea; 1% (wt/vol) sodium dodecyl sulfate; 1% (vol/vol) β-mercaptoethanol; 0.05 μM Tris, pH 7.0; and 0.01% (wt/vol) phenylmethylsulfonylfluoride. Conditioned media OPG and RANKL secretion and cell-associated RANKL levels were determined using the QuantiTikine M mouse OPG and mouse sRANKL enzyme-linked immunoassays available from R&D Systems (Minneapolis, MN) following the manufacturer's directions. These enzyme-linked immunoassays detect unbound proteins without interference from the OPG/RANKL complex. Absorbance was measured at 450 nm with a correction wavelength set at 540 nm.

**Transient transfection and luciferase assay**

CREB wild-type and mutant oligodeoxynucleotides (ODN) (Gene Detect, New Zealand) decoy transcription factor binding sites were transected into 70–80% confluent MC3T3-E1 cells. Transient transfections were performed using 6 μl of FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) per 35-mm well according to manufacturer’s instructions. Briefly, cells were plated in 24-well cell culture plates and grown for 48 h. The cells were then cultured for 24 h in serum-free, penicillin/streptomycin-free media. Three and a half μM of mutant or wild-type ODN and 400 ng pRL-TK plasmid (Renilla luciferase driven by thymidine kinase promoter) were incubated for 1 h at room temperature with FuGENE 6 in 100 μl of serum-free, antibiotic-free media. The lipd/ODN mixture was added drop-wise to the cell cultures to a final volume of 200 μl. The cells were allowed to incubate for 24 h, and then the media was collected and OPG levels were measured. Luciferase assays were performed using Dual Luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer’s instructions and MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA).

Secreted alkaline phosphatase was measured from media obtained from cultured MC3T3-E1 cells transfected with the pCRE-secreted alkaline phosphatase (SEAP) plasmid. Transient transfections were performed as described above. Briefly 1.6 μg of pCRE-SEAP plasmid DNA (Clontech), 400 ng pRL-TK, and 3.1 μg FuGENE 6 were added to each well of MC3T3-E1 cells grown to 50% confluence in six-well plates. After 12 h, cells were switched to serum-free α-MEM. After 12 h in serum free medium, the media were removed and fresh media were added that contained 1 μM nifedipine and 10 mM gadolinium (Sigma) or vehicle. The cells were incubated for 30 min with the inhibitors before addition of 10 nM 1,25(OH)2D3 or vehicle (0.01% EtOH). Media were removed at 0, 3, and 6 h post treatment. Cells were lysed and SEAP and luciferase activity were measured in the same manner as described above. All assays were measured in triplicate wells and normalized to luciferase activity.

**Statistical analysis**

All values are expressed as mean ± sp. Real-time RT-PCR assays were performed in triplicate and the entire experiment repeated three times using two independent RNA isolates. Enzyme linked immunoassays were performed on MC3T3-E1-conditioned media from triplicate wells and repeated with a later cell passage. OPG measurements from conditioned media obtained from cultured calvaria were performed in duplicate and confirmed using calvaria isolated from a second litter of pups. Transient transfections were carried out in triplicate and repeated twice. Statistical comparisons were made between treatment and control groups using the t test with Dunn’s posttest. Fold changes referred to in the text were calculated by dividing the value of the treatment group by the value of the control sample at the same time point. For each experiment, OPG and sRANKL data were normalized by arbitrarily setting the value for test protein in the medium of vehicle-treated MC3T3-E1 cells at time 0–1.0, essentially background.

**Results**

**OPG and RANKL protein secretion and mRNA expression in MC3T3-E1 cells and primary calvarial cultures**

ELISA-based protein measurements for OPG and sRANKL were performed using conditioned media collected from MC3T3-E1 cells and cultured mouse calvaria to determine whether 1,25(OH)2D3 affects OPG and sRANKL protein secretion from osteoblastic cells. Media concentrations of OPG and sRANKL from calvarial cultures were compared with those from MC3T3-E1 cells to determine whether the osteoblastic cell line responded to 1,25(OH)2D3 in the same manner as the organ culture. In untreated MC3T3-E1 cells, which were used to standardize the ELISA data, the baseline secretion levels reflecting 1 d accumulation in culture were 75.2 pg/ml for OPG, 7.2 pg/ml for sRANKL, and 16.4 pg/ml for mRANKL. Treatment with 20 nM 1,25(OH)2D3 for 24 h resulted in a reduction in OPG in the culture media for both MC3T3-E1 cells and mouse calvaria (Fig. 1, A and C). Differences in OPG secretion in both cultures systems were not detected until after 6 h treatment with 1,25(OH)2D3 when compared with vehicle treatment. At 24 h, OPG secretion from MC3T3-E1 cultures was reduced to 0.47 of the vehicle-treated control, whereas cultured calvarial secretion of OPG into the conditioned media was 0.43 of the vehicle-treated control levels at the same time point. sRANKL was measured in the same conditioned media that was used to determine media concentrations of OPG (Fig. 1, B and D). 1,25(OH)2D3 treatment did not affect sRANKL secretion by cultured calvaria until after 6 h. Twenty-four hours after application of 1,25(OH)2D3 to calvarial cultures, there was a 2.5-fold increase in the amount of sRANKL in the media from 1,25(OH)2D3-treated vs. vehicle-treated control calvaria. Unlike cultured calvaria, secretion of sRANKL by MC3T3-E1 cells remained at baseline levels at all time points examined (Fig. 1B). mRANKL levels in 1,25(OH)2D3-treated cells did not differ from vehicle-treated MC3T3-E1 cells at any time point examined (data not shown).

OPG and RANKL mRNA expression were measured by real-time RT-PCR in murine MC3T3-E1 cells that were grown to 80% confluency and treated for 24 h with either 20 nM 1,25(OH)2D3 or vehicle (0.01% EtOH). As shown in Fig. 2A, OPG mRNA expression decreased to 0.66 the level of vehicle-treated controls by 3 h in MC3T3-E1 cells treated with 1,25(OH)2D3. At 6 h, 1,25(OH)2D3 treatment decreased OPG expression to 0.10 the level of 6 h vehicle-treated control, and this decrease persisted through the later time points. OPG did not decrease in MC3T3-E1 cells treated for 24 h with vehicle. MC3T3-E1 cells expressed detectable levels of mRNA for RANKL; however, 1,25(OH)2D3 did not affect RANKL mRNA levels when compared with vehicle-treated controls (Fig. 2B). This low level of mRNA encoding RANKL probably explains the detection of mRANKL seen by ELISA.

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The untreated MC3T3-E1 cells showed 3.2 times more mRNA encoding OPG than that encoding RANKL, a finding consistent with that seen previously by others and with our ELISA data (36).

**Regulation of OPG secretion by VSCCs**

We previously showed that 1,25(OH)2D3 alters Ca2+ permeability in osteoblastic cells within seconds of treatment by modulating the activity of VSCCs (16, 17). To determine whether the decreases in OPG secretion and mRNA levels seen in MC3T3-E1 cells were associated with altered Ca2+ permeability, we treated MC3T3-E1 cells with specific inhibitors of VSCCs to determine whether changes in OPG secretion levels required Ca2+ influx across the plasma membrane. MC3T3-E1 cells were pretreated for 30 min with specific inhibitors to the L-, T-, P/Q-, N-, and R-type VSCCs. The cells were then rinsed and fresh media containing either VSCC inhibitor or vehicle (0.01% EtOH with 0.1% DMSO) were added. Media were collected at 0, 3, 6, 12, and 24 h after application of VSCC inhibitors or vehicle and then analyzed for OPG concentrations by ELISA. Addition of nifedipine, a specific inhibitor of the L-type VSCC, reduced OPG secretion into the media to 0.55 the level of vehicle-treated cells (Fig. 3). The decrease in OPG secretion was first detectable at 3 h and persisted through 24 h, although a slight rebound was apparent at the 24-h time point. Blockade of the T-, P/Q-, N, and R-type VSCCs did not affect OPG levels secreted into the media (Fig. 3). Furthermore, OPG secreted into the culture media by cells treated with 20 nM 1,25(OH)2D3 in the presence of BAY K 8644 was not significantly different from MC3T3-E1 cells treated with 1,25(OH)2D3 alone (data not shown). Likewise, OPG secretion by cells treated with 20 nM 1,25(OH)2D3 along with nifedipine was similar to nifedipine alone (Fig. 3) except that the line remained essentially flat at 24 h when both compounds were present. Taken together, the experiments in Figs. 1–3 suggested that the 1,25(OH)2D3-induced decrease in OPG secretion might occur through a mechanism involving Ca2+-dependent inactivation of L-type VSCCs whose activity normally maintains OPG transcription.
FIG. 3. OPG secretion by MC3T3-E1 cells is regulated by VSCC activity. Cells were pretreated for 30 min with the indicated VSCC inhibitors, and then the media were changed and fresh media containing VSCC inhibitors were added. Media were collected at the indicated time points and OPG levels were measured. Inhibiting the L-type VSCC with 1 μM nifedipine (●) reduces OPG secretion into the media at 24 h. Cotreatment with 1 μM nifedipine and 20 nM 1,25(OH)2D3 (●) decreases OPG secretion similarly to cells treated with nifedipine alone but does not begin to rebound at 24 h. Inhibiting the T-type VSCC with 200 nM sTX-3.3 (●), P/Q-type VSCC with 1 μM ω-agatoxin IVA (●), N-type VSCC with 1 μM ω-conotoxin GVIA (●), and R-type VSCC with 100 nM SNX-482 (●) did not affect OPG secretion when compared with vehicle-treated cells (□). Note that this experiment represents a composite of normalized data from several reproducible experiments using various combinations of these treatments. *** P < 0.001, treated, compared with vehicle-treated cultures at the same time point.

**CaV1.2 expression in MC3T3-E1 cells**

In addition to modulating Ca2+ permeability of the osteoblast plasma membrane, 1,25(OH)2D3 also reduces L-type VSCC biosynthesis in osteoblastic cells providing a mechanism for cross-talk between genomic and nongenomic pathways (12). To determine whether long term down-regulation of CaV1.2 might account for the sustained reduction in OPG secretion we observed, mRNA quantification and immunohistochemistry were performed on 80% confluent MC3T3-E1 cells to measure the changes in CaV1.2 protein levels and mRNA expression after 24 h treatment with 20 nM 1,25(OH)2D3 or vehicle (0.01% EtOH) (Fig. 4). Real-time RT-PCR was performed using CaV1.2 sequence-specific primers on 1,25(OH)2D3 and vehicle-treated cells (Fig. 4). L19 used as load control to normalize mRNA expression levels (data not shown). By 6 h, the amount of CaV1.2 mRNA in 1,25(OH)2D3-treated cells was 0.41 that of vehicle-treated cells. mRNA levels further decreased to 0.25 and 0.10 at 12 and 24 h, respectively, a greater difference than seen previously in ROS (17/2.8 cells (37)). An increase in the levels of CaV1.2 mRNA was seen in the vehicle-treated cells as they withdrew from the cell cycle and began to differentiate in serum-free medium. This increase is similar in magnitude to increases seen for other osteoblastic markers including osteopontin and collagenase-3 that also increase during this time period (data not shown).

Immunohistochemistry revealed that protein levels of CaV1.2 also decreased by 24 h (Fig. 4B). Cells treated for 24 h with vehicle displayed strong antibody staining for CaV1.2 on the cell surface and also in regions of the cell in close proximity to the nucleus, likely representing protein present in the synthetic route of the endoplasmic reticulum and Golgi complex. Treatment with 1,25(OH)2D3 decreased staining for CaV1.2. Staining close to the nucleus was still present but not as bright as detected in vehicle-treated cells, and the staining for CaV1.2 in the cell periphery has significantly diminished.

**CaMK, PKA, and MAPK regulation of OPG secretion**

Alterations in local intracellular Ca2+ levels regulate activity of several second messenger systems through activation or inactivation of protein kinases. We used pharmacological inhibition of protein kinases to determine whether 1,25(OH)2D3-mediated changes in OPG expression involved activity of CaMK, PKA, and MAPK. For this, MC3T3-E1 cells were treated with specific inhibitors of these potentially Ca2+-regulated signaling pathways (Fig. 5). MC3T3-E1 cells

**FIG. 4. CaV1.2 expression in MC3T3-E1 cells in response to treatment with 20 nM 1,25(OH)2D3.** A, mRNA levels for CaV1.2 decreased in response to 1,25(OH)2D3 (data ± SD). B, Confocal images of MC3T3-E1 cells using a rabbit antimouse primary antibody to CaV1.2 and FITC-conjugated donkey antirabbit IgG (green) and counterstained with the nuclear dye ToPro-3 (red). ***, P < 0.001, **, P < 0.01, 1,25(OH)2D3-treated, compared with vehicle-treated cultures at the same time point.

**FIG. 5. CaMK, MAPK, and PKA regulation of OPG secretion.** Cells were pretreated for 30 min with vehicle (0.01% EtOH with 0.1% DMSO) (●) or inhibitors to CaMK with KN93 (●), MAPK pathway with PD98059 (●), or PKA with H89 (●). After the incubation, the media were changed and fresh media containing additional kinase inhibitors were added. Media were collected at the indicated time points and OPG levels were measured. ***, P < 0.001, CaMK inhibitor cells, compared with vehicle-treated cultures at the same time point.
treated with KN93, a specific inhibitor of CaMKs, secreted 0.63 the levels of OPG, compared with vehicle control cells at 12 h, and this was further reduced to 0.55 by 24 h when compared with vehicle-treated control cells. Application of the MAPK pathway inhibitor PD98059 decreased OPG secretion to 0.83 the levels of vehicle control cells at both 12 and 24 h. OPG secreted by MC3T3-E1 cells treated with H89, a PKA inhibitor, did not differ from vehicle-treated cells at the same time points.

To elucidate whether the observed changes in OPG found in the culture media reflected decreased protein secretion or lower levels of transcription, real-time RT-PCR was performed on 80% confluent MC3T3-E1 cells treated with inhibitors to the L-type VSCC, CaMK, MAPK pathway, and PKA and vehicle (0.01% EtOH with 0.1% DMSO) for 24 h (Fig. 6). Blocking the L-type VSCC with nifedipine reduced OPG mRNA expression to 0.66 the levels of vehicle-treated controls. OPG mRNA levels were 0.32 the value of vehicle-treated control cells when CaMK was inhibited by KN93, a 3.1-fold decrease. Treatment of MC3T3-E1 cells for 24 h with inhibitors to PKA and MAPK pathway had no significant effect on OPG mRNA expression.

**OPG secretion by cultured mouse calvaria cultures treated with inhibitors to CaMK and the L-type VSCC**

Mouse calvaria were treated with nifedipine and KN93 to verify that the changes in OPG secretion by MC3T3-E1 cells in response to blocking the L-type VSCC and CaMK were applicable to a more physiologically complete system. Calvaria treated for 24 h with 20 nM 1,25(OH)2D3 displayed a reduction in OPG secretion to 0.38 the levels of vehicle (0.01% EtOH with 0.1% DMSO)-treated cultures (Fig. 7). Similar to the MC3T3-E1 cells, blocking the L-type VSCC in calvarial mouse calvaria resulted in a reduction of OPG secretion to 0.66 the levels of vehicle-treated controls at 12 and 24 h, respectively. Treating calvaria for 24 h with the CaMK inhibitor KN93 resulted in a reduction of OPG secretion to 0.31 the levels of vehicle-treated controls.

**CRE regulation of OPG expression**

The transcription factor CREB is a potential effector of L-type VSCC-dependent changes in OPG transcription elicited by 1,25(OH)2D3. Analysis of the OPG promoter revealed that there are putative consensus CRE-binding sites at -563 in the mouse promoter and at -289 and -104 in the human promoter. CREB activity is regulated by protein kinases including CaMK, PKA, and MAPK pathways in other cellular systems (see Discussion). To determine whether CaMK was affecting OPG expression by acting on a CRE-binding element, MC3T3-E1 cells were transiently transfected with constructs containing wild-type or mutant consensus CRE-binding sites (Fig. 8). The cells were transfected with 1μM wild-type CRE decoy receptor or 1μM mutant CRE decoy receptor or vehicle (0.01% EtOH and 3% FuGENE 6). Twenty-four hours after transfection, the media were collected and analyzed for OPG in the conditioned media. MC3T3-E1 cells transfected with 1μM of the wild-type decoy CRE-binding site secreted 0.26 the levels of OPG into the media as did vehicle-treated cells. One micromolar concentration of the mutant decoy CRE-binding site did not alter the levels of OPG secreted into the culture media.

To more directly evaluate the role of L-type VSCC in regulating CRE activity, MC3T3-E1 cells were transiently transfected with the pCRE-SEAP plasmid that contains CRE elements immediately upstream of a reporter cDNA. For this, MC3T3-E1 cells were transiently transfected with pCRE-SEAP and the effect of blocking the L-type VSCC was assayed (Fig. 9). The reporter cDNA in pCRE-SEAP plasmid is the secreted alkaline phosphatase providing a sensitive assessment of CRE activity. After blockade of the VSCC or treatment with vehicle (0.01% EtOH), secreted
Percent confluent MC3T3-E1 cells in serum-free medium were transiently transfected with CRE wild-type and mutant oligodeoxynucleotides. The cells were treated with vehicle (0.01% EtOH and 3% FuGENE 6), 1 μM wild-type CRE decoy receptor, or 1 μM mutant CRE decoy receptor. Twenty-four hours after transfection, the media were collected and analyzed for OPG levels. The wild-type CRE decoy receptor, but not the mutant, reduced OPG secretion into the media.

**Discussion**

OPG and RANKL are key regulators of osteoclastogenesis and are predominantly expressed by immature osteoblastic cells. During periods of low-serum Ca\(^{2+}\), 1,25(OH\(_2\))\(_D_3\) acts to increase the RANKL/OPG ratio and thus promote activation and maturation of osteoclast progenitors, facilitating liberation of stored Ca\(^{2+}\). In this study, we found that 1,25(OH\(_2\))\(_D_3\) treatment decreased OPG secretion, first detectable at 6 h, a time when numerous transcriptional changes have clearly occurred (27). OPG secretion from MC3T3-E1 cells treated for less than an hour with 1,25(OH\(_2\))\(_D_3\) did not differ from the secreted levels of OPG found in vehicle-treated cultures at the same time points, suggesting that the mechanism of down-regulation is unlikely to merely involve a shutdown of stimulus-secretion coupling that would have been manifest much faster. OPG mRNA and protein levels were decreased by application of 1,25(OH\(_2\))\(_D_3\) in both primary organ cultures and osteoblastic cell lines, with the mRNA reduction seen before the decrease in protein secretion. In contrast to OPG, 1,25(OH\(_2\))\(_D_3\) increased sRANKL secretion by calvarial cultures over a similar time frame. Consistent with their poor ability to support osteoclastogenesis, MC3T3-E1 cells produced little or no sRANKL regardless of whether 1,25(OH\(_2\))\(_D_3\) was present, although they did have a small, but detectable, amount of mRNA encoding RANKL. From this result, we concluded that MC3T3-E1 cells provide appropriate models for in vitro study of OPG expression and secretion but not for RANKL. For this reason, the majority of our mechanistic studies focused on OPG.

Interestingly, in MC3T3-E1 cells, the transcript levels encoding OPG decreased at 3 h after addition of 1,25(OH\(_2\))\(_D_3\), before the decrease in protein secretion, suggesting a transcriptional change that precedes the change in protein secretion. Changes in the rate of translation, posttranslational modifications, and alterations in the secretory pathway could play additional roles in the changes in OPG secretion after 1,25(OH\(_2\))\(_D_3\) treatment, and are of interest for future study. Nonetheless, the effects of 1,25(OH\(_2\))\(_D_3\) treatment that we observed at the mRNA level at 3 h prompted us to examine potential mechanisms further.

Ca\(^{2+}\) is a common second messenger and plays a role in intracellular contraction, protein secretion, and gene transcription (23). Previous in vivo results demonstrated that administration of small doses of the L-type VSCC blocker nifedipine twice daily for 10 wk to growing rabbits resulted in reduced epiphysial growth plate thickness and mineral apposition rates (38), implicating the L-type VSCC as a player in bone development and modeling. Previous studies from our group and others have shown that the L-type VSCC, Ca\(_{V1.2}\), is the predominant Ca\(^{2+}\) channel present in proliferating osteoblasts (16, 17, 39). In this study, blocking the activity of L-type VSCCs in MC3T3-E1 cells and primary calvarial organ cultures reduced the level of OPG protein secretion by 3 h, considerably faster than did 1,25(OH\(_2\))\(_D_3\). When 1,25(OH\(_2\))\(_D_3\) was present along with dihydropyridine, the cells did not recover OPG secretion even over a 24-h period. Specificity was shown by the observation that blocking other families of VSCCs with specific inhibitors did not
CaV1.2 subunit of the L-type VSCC is down-regulated after 1,25(OH)2D3 treatment (0.1–100 nm) when examined by real-time RT-PCR using the same RNA sets that were used to determine CaV1.2 expression in Fig. 4, confirming that the changes in CaV1.2 mRNA levels were due to specific transcriptional changes. This long-term down-regulation in CaV1.2 biosynthesis provides a molecular mechanism to explain the longer-term repression of OPG production by 1,25(OH)2D3, than seen with nifedipine alone in Fig. 3. We suggest that OPG production depends on the activity and expression of CaV1.2 and that the loss of OPG mRNA at 3 h after 1,25(OH)2D3 treatment reflects a Ca2+-dependent signaling feedback response, whereas the longer-term down-regulation after 6–12 h reflects the reduction in VSCC levels (Fig. 10). Because analysis of the OPG promoter revealed no consensus vitamin D response element, it seems likely that the 1,25(OH)2D3 reduction in OPG expression and secretion does not involve a direct action of the nuclear vitamin D receptor.

The differences in time frame suggest that down-regulation of OPG and CaV1.2 transcription occurs by independent mechanisms. When examined electrophysiologically (16), we found that increases in Ca2+- entry by 1,25(OH)2D3 are short lived in the absence of other depolarizing stimuli, such as PTH. As we noted previously, inactivation of L-type channels is influenced by intracellular Ca2+ levels in proximity to the channel. Thus, steady-state inward current is dependent on current amplitude and rates for buffering intracellular Ca2+. Pharmacological inhibition of the L-type channel by dihydropyridines and Ca2+-induced inactivation of VSCCs caused by 1,25(OH)2D3 both reduce VSCC activity, but nifedipine acts faster and does not block the longer-term effects associated with Ca2+-independent reduction in CaV1.2 expression (37). A current focus of our laboratory is to identify the components of the short-term feedback mechanism that modulates VSCC activity and based on parallels with excitable cells is likely to involve phosphorylation of channel subunits. Regardless, down-regulation of OPG secretion by 1,25(OH)2D3 is a Ca2+-influx-dependent process that could require rapid changes in transcription or activity of protein kinases. Considered in conjunction with the decrease in OPG expression and secretion obtained by inhibiting the L-type VSCC with nifedipine, these data demonstrate that the activity of the L-type VSCC is necessary for basal OPG synthesis and secretion and that the reduction in OPG mRNA and protein observed at 6–12 h post treatment with 1,25(OH)2D3 is at least partially attributed to 1,25(OH)2D3 down-regulating CaV1.2 transcript levels and hence further reducing Ca2+- influx rates. We point out that at these longer time points, other signaling pathways that regulate OPG gene expression are certainly operational and provide an opportunity to examine cross-talk between Ca2+-dependent and independent pathways in osteoblasts.

Our data showed that inhibition of CaMK with KN93 resulted in decreased OPG mRNA expression in MC3T3-E1 cells and reduced secretion of OPG in both organ cultures...
and osteoblastic cell lines. Additionally, MC3T3-E1 cells treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} along with inhibitors to CaMK, PKA, MAPK, or \textit{CaV}_{1.2} have secreted OPG levels equal to 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated cultures by 24 h and significantly lower levels than vehicle-treated cultures treated with the same inhibitors, suggesting that the 1,25(OH)\textsubscript{2}D\textsubscript{3} effect predominates at the time the nuclear receptor down-regulation of \textit{CaV}_{1.2} has occurred (data not shown). The shorter term down-regulation, however, remains of mechanistic interest. Calmodulin (CaM), a major intracellular Ca\textsuperscript{2+} receptor, has been reported to be tethered to the C-terminal tail of \textit{CaV}_{1.2} (45) as well as free in the cytosol. CaM localization to the L-type VSCC may explain the Ca\textsuperscript{2+} influx requirement for maintenance of OPG secretion. Initiation of PKA signaling pathways typically requires hormone binding to its receptor to generate cAMP (46). Our data suggest that inhibition of PKA does not significantly affect OPG expression or secretion, consistent with data indicating that PTH-mediated increases in PKA signaling augment Ca\textsuperscript{2+} responses (17). Makiishi-Shimobayashi et al. (47) suggested that OPG expression is regulated through the Ras/MAPK signaling cascade due to increased phosphorylation of ERK-1 and ERK-2 in response to IL-18 treatment. However, our data indicated that inhibition of MAPK pathway with PD98059 did not significantly affect OPG transcript or protein secretion in MC3T3-E1 cells, suggesting that the increase in phosphorylation of ERK-1 and ERK-2 may be coincidental. Taken together, our data support the idea that CaMK signaling associated with L-type VSCC activity is a primary mechanism to maintain OPG secretion in osteoblasts.

CaM interacts with and regulates various proteins including calcium channels, CaMK, calcineurin, and activator protein-1, all of which can regulate transcription (23, 48, 49). CaMK can modulate gene expression through the function of
the CRE promoter element (28, 49). There are four major classes of CaMK, and MC3T3-E1 cells express mRNA for CaMKI, CaMKII, and CaMKIV (data not shown). CaMKIV can translocate into the nucleus and phosphorylate ser-133 in CREB, promoting binding to CRE and modification of gene transcription (50). The OPG promoter has putative consensus CRE sites at −563 in the mouse promoter and at −289 and −104 in the human promoter. In these studies, transfection of decoy CRE binding sites for CREB reduced OPG secretion by competing away the phosphorylated transcription factor, whereas transfection with mutant CRE decoy binding sites did not affect OPG secretion, suggesting that CaMK regulation of OPG expression and secretion involves activity of CREB/CRE. We do not know, however, whether the phosphorylated CREB affects gene transcription by binding directly to the CRE elements in the OPG promoter or whether CREB binds to and down-regulates a negative regulatory factor of OPG. Further studies are needed to clarify the mechanism by which OPG mRNA is regulated by CREB in osteoblastic cells.

One important aspect of this study is that it provides a novel physiological function for the VSCC in osteoblastic cells in maintaining secretion of OPG and possibly RANKL. This new level of regulation occurs on a backdrop of other mechanisms that also can inhibit OPG expression. We present a model through which the Ca2+ signal generated by the regular activity of the L-type VSCC maintains basal OPG expression and secretion through a CaMK-mediated pathway (Fig. 10). In the context of the reduction of OPG secretion associated with 1,25(OH)2D3, two temporally distinct mechanisms of down-regulation are revealed. We propose that as 1,25(OH)2D3 levels increase in response to low serum Ca2+, CaV1.2 activity in osteoblasts decreases first by a Ca2+/CaMK-dependent signaling feedback mechanism that is triggered by the initial increase in Ca2+ influx in response to 1,25(OH)2D3. This short-term negative feedback mechanism (Fig. 10A) is simulated by pharmacological blockade of the VSCC by nifedipine and accounts for the reduction in OPG secretion seen first at 6 h as shown in Fig. 7. If 1,25(OH)2D3 levels remain high, Ca2+ influx capacity of the cell further decreases as CaV1.2 biosynthesis decreases and channel numbers are reduced (Fig. 10B). Reductions in local Ca2+ influx through VSCCs by either mechanism diminish the activation of CaMK and the subsequent phosphorylation of CREB, but the time courses are distinct. When OPG basal production drops, there follows a shift in the OPG:RANKL ratio, favoring osteoclastogenesis and allowing the release of stored Ca2+ from bone. In the longer term, it will be of interest to mechanistically examine the exact mechanisms of down-regulation of OPG production by 1,25(OH)2D3 including the potential for VSCC cross-talk with signaling pathways such as those activated by other cytokines and calcitropic hormones.

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