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The calcium-sensing receptor (CaR) is involved in strontium ranelate-induced osteoblast proliferation

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ABSTRACT

Strontium ranelate has several beneficial effects on bone and reduces the risk of vertebral and hip fractures in women with postmenopausal osteoporosis. We investigated whether Sr^{2+} acts via a cell surface calcium-sensing receptor (CaR) in HEK293 cells stably transfected with the bovine CaR (HEK-CaR) and rat primary osteoblasts (POBs) expressing the CaR endogenously. Elevating Ca_o^{2+} or Sr^{2+} concentration-dependently activated the CaR in HEK-CaR but not in non-transfected cells, but the potency of Sr^{2+} varied depending on the biological response tested. Sr^{2+} was less potent than Ca_o^{2+} in stimulating inositol phosphate accumulation and in increasing Ca_i^{2+}, but was comparable to Ca_o^{2+} in stimulating ERK phosphorylation and a non-selective cation channel, suggesting that Ca^{2+} and Sr^{2+} have differential effects on specific cellular processes. With physiological concentrations of Ca_o^{2+}, Sr^{2+}-induced further CaR activation. Neither Sr^{2+} nor Ca_o^{2+} affected the four parameters just described in non-transfected cells. In POB, Sr^{2+} stimulated cellular proliferation. This effect was CaR-mediated, as transfecting the cells with a dominant negative bovine CaR significantly attenuated Ca_o^{2+}-stimulated POB proliferation. Finally, Sr^{2+} significantly increased the mRNA levels of the immediate early genes, c-fos and egr-1, which are involved in POB proliferation, and this effect was attenuated by overexpressing the dominant negative CaR. In conclusion, Sr^{2+} is a full CaR agonist in HEK-CaR and POB, and, therefore, the anabolic effect of Sr^{2+} on bone *in vivo* could be mediated, in part, by the CaR.

1. Introduction

Strontium ranelate (PROTELOS®) is a new chemical entity containing two atoms of stable strontium (Sr^{2+}). Strontium ranelate has been proposed as a preventive and curative treatment of osteoporosis that reduces the risk of both vertebral and non-vertebral fractures in postmenopausal women [1–3]. Beneficial effects of strontium ranelate have also been reported in various animal models, where it has been shown to prevent bone loss by increasing bone formation and decreasing bone resorption [4–9]. These *in vivo* results were consistent with *in vitro* data where strontium ranelate has been shown to reduce bone resorption by osteoclasts and to stimulate bone formation by osteoblasts (OBs) [10–12].

The mechanisms of action of strontium ranelate at the cellular level have not yet been fully elucidated. Since Sr^{2+} is a divalent cation that closely resembles Ca^{2+} in its atomic and ionic properties, we hypothesized that Sr^{2+} is an agonist of the extracellular calcium-sensing receptor (CaR), a G protein-coupled receptor first cloned from parathyroid gland [13]. The

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physiological importance of the CaR has been demonstrated by the existence of several human hyper- or hypocalcemic disorders arising from inactivating or activating mutations, respectively, in its coding region of the CaR gene [for review 14]. The CaR is also expressed in many other tissues, e.g., intestine, brain, kidney and bone (e.g., in OBs and osteoclasts), where its functions are less well known [for review 15].

Strontium ranelate has been shown to reduce osteoclast differentiation and activity [11–12] and to stimulate cellular proliferation of pre-OBs, leading to an increased number of OBs, thereby enhancing matrix deposition and bone tissue formation [10]. The molecular identity of the Ca^{2+}-sensing mechanism(s) that mediates the effects of Ca^{2+} on OBs remains much debated. Expression of an additional putative calcium sensor in OBs has been reported, which has been proposed as a mediator of the actions of high extracellular calcium on osteoblasts [16]. Nevertheless, the CaR has been shown to be expressed in several osteoblastic cell lines [17,18]. Not only is the CaR present in several osteoblastic and stromal cell lines [19], but it is also expressed in intact bone [20]. In sections of murine, rat and bovine bone, CaR mRNA and protein are expressed in *bona fide* OBs, as assessed by *in situ* hybridization and immunohistochemistry with specific anti-CaR probes and antibodies, respectively [20]. In addition, the CaR is expressed at all stages of OB development, from the proliferating, preosteoblastic stage to the more mature, bone-forming stage of OBs in culture [21].

It has been shown that the CaR expressed in OBs exerts several actions on bone cells *in vitro* [17,18,21,22] that could be the basis for the known anabolic effects of Ca_o^{2+} in intact bone. Preliminary data have demonstrated that the CaR recognizes not only Ca_o^{2+} but also Sr^{2+} [23–25]. Thus, this receptor is a candidate for mediating the anabolic actions of Sr^{2+} on bone.

The goal of the present study was first to evaluate the capacity of Sr^{2+} (in the form of strontium ranelate) to directly activate the CaR, as assessed by measuring the activation of phospholipase C (PLC), elevation in the cytosolic-free calcium concentration (Ca_i^{2+}), accumulation of inositol phosphates (IPs) and activation of a non-selective cation channel (NCC) in human embryonic kidney (HEK293) cells transfected with the bovine CaR. The results indicate that Sr^{2+} is an agonist of the CaR, whose potency and efficacy are similar to those of calcium for some parameters of cellular function and less for others. The role of the CaR in mediating the stimulatory action of Sr^{2+} on cell proliferation was then examined in rat primary osteoblasts (POBs), along with the underlying mechanism. The results show that Sr^{2+} increases cell proliferation as well as the expression of immediate early genes associated with cell proliferation, and that expression of the functional CaR is required for mediating these effects.

2. Materials and methods

2.1. Tested products

Strontium ranelate (Technologie Servier, Orléans, France) was used to test the activity of Sr^{2+} on the CaR. Due to limited solubility of strontium ranelate in the culture medium, which precluded the use of Sr^{2+} concentrations higher than 3 mM, $SrCl_2$ (APS grade, Sigma St. Louis, MO, U.S.A,), mixed with sodium ranelate (Technologie Servier, Orléans, France), was used to test levels of Sr^{2+} up to 10–15 mM. A 1/100 ratio between ranelic acid and Sr^{2+} was used to mimic the therapeutic circulating ratio. As no appreciable differences in the results were obtained using both products, results are simply expressed in terms of the divalent strontium ion (Sr^{2+}). Calcium chloride (SigmaUltra, Sigma, St. Louis, MO, U.S.A.) was used as a source of Ca^{2+} and served as a positive control for agonist activity in all experiments.

2.2. Transiently and stably transfected HEK293 cells

Human embryonic kidney (HEK293) cells were obtained from ATCC (Manassas, VA, USA). A clonal HEK293 cell line stably transfected with the cDNA for the bovine CaR was generated by transfecting HEK293 cells with the cDNA of the bovine CaR in the pcDNA3 vector and selecting based on hygromycin resistance. It was previously established that non-transfected HEK293 cells do not express an endogenous CaR. Only transfected HEK293 cells (HEK-CaR) express the CaR on their cell surface and are responsive to addition of CaR agonists to the external medium [26,27]. In our previous experience, stably transfected HEK293 cells exhibit consistent biochemical and morphological properties through at least 15 passages. Thus, the experiments performed in this study were performed on cells within the first 15 passages after transfection.

Some studies, e.g., the effects of Ca_o^{2+} and Sr^{2+} on intracellular Ca^{2+} mobilization, were performed using HEK293 cells transiently transfected with the same construct of the bovine CaR used for stable transfection. Transient transfection was carried out on cells that were grown to 80–90% confluence in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, U.S.A.) using lipofectamine as described previously. Functional characterization of CaR activity in the cells was performed 36–48 h after transfection.

Transiently or stably transfected cells were grown in DMEM with 10% fetal bovine serum, without sodium pyruvate, either without (wild-type HEK293), or with 200 μg/mL hygromycin (Boehringer-Mannheim) (HEK-CaR). Prior to experimental manipulations, subconfluent cell monolayers were serum-starved in DMEM supplemented with 0.2% BSA (cell culture tested, Sigma, St. Louis, MO, U.S.A.) for 24 h. After aspiration of the culture medium, the cells were incubated for varying times with the concentrations of Ca_o^{2+} or Sr^{2+} detailed in the Results section.

2.3. Calvarial OB (POB) cultures

For each experiment, about 25–30 calvariae were harvested at room temperature from 21-day (E21) fetal rats (*Sprague Dawley*). Humane handling of rats was carried out according to the guidelines of the Center for Animal Resources & Comparative Medicine of Harvard Medical School. A previously described method of repeated digestion of the calvariae with 0.05% trypsin and 0.1% collagenase P (Roche, Indianapolis, IN) was used to release cells [21]. After discarding

the cells from the first two digestions, cells from the next three digestions were pooled and cultured in DMEM containing 10% heat-inactivated FBS and 1% penicillin–streptomycin in 5% CO_2 at 37 °C. All routine culture media were obtained from Invitrogen (Grand Island, NY). For the proliferation assay and mRNA analyses, cells were plated on 96-well and 24-well plates, respectively. Routinely, cultures were maintained for 2–3 days after plating, at which stage they are 70–80% confluent. Cells were then serum-starved in DMEM (0.5 mM Ca^{2+}, 4 mM L-glutamine, 1% penicillin–streptomycin and 0.2% BSA) for 4 h before all experiments. All experiments were carried out within 7 days after beginning the culture.

2.4. Measurement of cytosolic-free calcium concentration

Measurement of cytosolic-free Ca^{2+} concentration (Ca_i^{2+}) was carried out using a cell population system [28]. HEK293 cells were cultured in DMEM with 10% fetal calf serum. Coverslips with near-confluent HEK293 cells (transfected or wild type) were loaded with 4 μM fura 2-acetoxymethyl ester (fura-2) (Molecular Probes, Eugene, OR, U.S.A.) for 2 h at room temperature in 1 mL of a buffer containing 130 mM NaCl, 4 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM HEPES, 1% dextrose and 1% BSA (pH 7.4 at 37 °C). The cells were washed in the same medium for 20 min and then placed diagonally into quartz thermostatted cuvettes equipped with a magnetic stirrer and containing the same medium except that the starting calcium concentration was 0 mM. Ca_o^{2+} or Sr^{2+} was then added in the increments shown in the Results section to achieve a final concentration of 9 mM. The experimental bath solution was stirred at 37 °C, and tested products were added at the desired final concentration. A dual wavelength xenon light source was used with a pair of excitation monochrometers centered at 340 and 380 nm. The emitted signal, filtered at 510 ± 40 nm, was collected at 90° with a photon counting photomultiplier tube (Photon Technology International, Princeton, NJ, U.S.A.). The ratio of emitted light at 340 nm excitation to that at 380 nm excitation was measured, and the normalized cumulative transient increases in Ca_i^{2+} were determined as the fraction of the maximal value observed with transfected HEK293 cells exposed to increasing concentrations of Ca_o^{2+} alone [29,30].

2.5. Measurement of inositol phosphate accumulation

HEK293 cells transiently or stably transfected with the bovine CaR were labeled with ^{3}H-inositol (~10 μCi/10^6 cells) overnight in medium 199 (with 1% penicillin–streptomycin, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5 and 15% bovine serum) [31]. They were then washed with medium (10 mM LiCl, 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$ and 2 mg/mL BSA in Eagle's solution), and incubated with varying concentrations of Ca_o^{2+} (0.5–10 mM) or Sr^{2+} (0–20 mM) for 30 min. At the end of the experiment, the reactions were terminated by the addition of a final concentration of 10% trichloroacetic acid (TCA). After sedimentation of precipitated debris (10,000 × *g*, for 10 min) and removal of the TCA by repeated extraction with diethyl ether, the neutralized supernatant was diluted with 1 mL distilled water and loaded on small polypropylene columns containing 0.8 mL dowex AG-1X8 (formate form, 200–400 mesh, Biorad, Richmond, CA, U.S.A.). Samples were then eluted sequentially with distilled water, 0.2, 0.4, 0.8 and 1.2 M ammonium formate in 0.1 M formic acid to separate ^{3}H-labeled IPs, e.g., IP_1, IP_2, IP_3 and IP_4. Ecosint (H (National Diagnostics, Atlanta, GA, U.S.A.) was added to the eluted fractions, and the radioactivity of the pooled IPs determined by liquid scintillation spectrophotometry.

2.6. Determination of ERK1/2 activity by Western blot

For the determination of extracellular signal-regulated kinase (ERK1/2) phosphorylation, monolayers of serum-starved transfected or non-transfected HEK293 cells were incubated at 37 °C in serum-free medium containing 0.2% BSA with varying levels of Ca_o^{2+} (0.5–10 mM) or Sr^{2+} (0–15 mM) [26]. At the end of the incubation period, the medium was removed, the cells were washed with ice-cold, phosphate-buffered saline (PBS) containing 1 mM sodium vanadate, and 100 μL of ice-cold lysis buffer was added as described previously [26]. The samples were then immediately frozen on dry ice. After thawing and homogenization, cell lysates were centrifuged at 10,000 × *g* for 5 min at 4 °C. Equal amounts of supernatant protein (10–15 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot analysis was performed as described below. The separated proteins on the gels were transferred electrophoretically onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.), and were incubated with blocking solution (10 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.05% Tween-20) containing 5% dry milk for 1 h at room temperature. ERK1/2 phosphorylation was then detected by incubating the membranes for 1–3 h with a 1:1000 dilution of a rabbit polyclonal, phospho-ERK1/2-specific primary antiserum (New England Biolabs, Bedford, MA, U.S.A.) and subsequently with a goat, anti-rabbit peroxidase-coupled, secondary antiserum diluted in blocking solution. The bands were visualized by chemiluminescence (Renaissance ECL system, Dumont-NEN, Boston, MA, U.S.A.). Quantitative comparisons of the phosphorylation of ERK1/2 under various experimental conditions were performed using an ImageQuant and a Personal densitometer (Molecular Dynamics). Nitrocellulose membranes were then stripped of antibodies and reprobed using an antiserum to ERK2 that detects this protein independently of its state of phosphorylation to confirm equal loading of ERK protein. Protein concentrations were measured using the micro BCA (Bicinchoninic acid) protein assay reagent kit (Pierce, Rockfod, IL, U.S.A.).

2.7. Measurement of non-selective cation channel activity by patch clamp

Channel activities were measured in cell-attached and inside-out patches using the patch clamp technique, as described previously [27]. The extracellular bath solution contained 140 mM NaCl, 4.0 mM KCl, 0.75 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10.0 mM glucose and 10.0 mM HEPES, pH 7.4. Solutions containing varying levels of Ca_o^{2+} or Sr^{2+} were applied to transfected or non-transfected HEK293 cells by superfusion. The pipette solution contained, unless noted otherwise, 87.0 mM NaCl, 55.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10.0 mM glucose and 10.0 mM HEPES, pH 7.4. When filled with

this external solution, pipette tip resistances were 5–10 MΩ. Currents were measured using an integrating patch clamp amplifier, and single channel currents were filtered at 3 kHz. Voltage stimuli were applied and single channel currents were digitized (200 μs per point) and analyzed using programs that were based on pClamp (Axon Instruments, Foster City, CA). The baseline current was monitored frequently to ensure proper analysis of single channel currents.

The probability of channel opening (P_o) was calculated from 30 s segments of current records using the equation: $P_o = I/Ni$, where I is the time-averaged current passing through the channels for a given period of time, N is the number of channels functioning independently within the membrane patch and i is the single channel current. Single channel measurements were taken at 0.5 mM Ca_o^{2+} and then following the addition of Ca_o^{2+} or Sr^{2+} to achieve the desired final concentrations in the culture medium.

2.8. Gene delivery by Raav

High efficiency gene transfer in OBs was accomplished using a recombinant adeno-associated virus (rAAV)-based method as described previously [21]. Both the bovine CaR sequence with a naturally occurring, dominant negative mutation R186Q (R185Q in the human CaR) [29] and the same vector containing the cDNA for the β-gal protein (as a negative control—referred to hereafter as BG) were under the control of a cytomegalovirus immediate-early (CMV-IE) promoter element and packaged as described previously. BG served as a control for any non-specific effects of rAAV infection. Cells were seeded (3–4×10^3 cells/well) in 96-well plates in 0.1 mL of growth medium and cultured overnight. For 24-well plates, 20,000 cells/well were seeded. About 1000 virus particles/cell (as optimized by pilot studies) were used to infect each well.

2.9. Quantitative real-time PCR

OBs were plated in 24-well plates and cultured for 2–3 days. SYBR green chemistry was used to perform quantitative determination of the relative expression levels of the transcripts for c-fos, egr-1 and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), following an optimized protocol described before [21]. The design of sense and antisense oligonucleotide primers was based on published cDNA sequences using Primer Express version 2.0.0 (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table 1. cDNA was synthesized with the Omniscript RT Kit (Qiagen, Valencia, CA) using 2 μg of total RNA in a 20-μL reaction volume. For real-time PCR, the cDNA was amplified using an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The dsDNA-specific dye SYBR Green I was incorporated into the PCR reaction buffer, QuantiTech™ SYBR PCR (Qiagen, Valencia, CA), to allow for quantitative detection of the PCR product in a 25-μL reaction volume. The temperature profile of the reaction was 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. An internal housekeeping control gene, GAPDH, was used to normalize differences in RNA isolation, RNA degradation and the efficiencies of the RT. The size of the PCR product was first verified on a 1.5% agarose gel, followed thereafter by melting-curve analysis.

Table 1 – PCR primers used for quantitation of egr-1 and c-fos transcripts by real time PCR

c-fos (X06769)	ATGTTCTCGGGTTTCAACGC (138) TGCGCAAAAGTCCTGTGTGT (287)
Egr-1 (NM_012551)	TGCTTCATCGTCTTCCTCTGC (802) TTGGGAGTAGGAAAGGTGGGT (909)
GAPDH	TTCAATGGCACAGTCAAGGC TCACCCCATTTGATGTTAGCG

2.10. Proliferation assay

Subconfluent calvarial OB cells on 96-well plates were serum starved for 4 h in Ca^{2+}-free DMEM containing 4 mM L-glutamine, 0.2% BSA and 0.5 mM $CaCl_2$. Cells were then stimulated in the same medium with various concentrations of Sr^{2+} (0.5–10 mM) for 18 h. Cell proliferation was measured using the BrdU ELISA from Roche according to the manufacturer's instructions [21]. For the last 3 h of the 18-h stimulation period, the cells were pulsed with BrdU. Absorbance at 450 nm was measured with a microplate reader (Model 550, Bio-Rad, Hercules, CA).

2.11. Statistics

The mean, standard error of the mean (S.E.M.) and the number of available values for each treatment were determined. Data were analyzed using one-way ANOVA followed by a Newman–Keuls multiple comparison test. For all statistical tests, a P-value < 0.05 was considered as statistically significant.

3. Results

3.1. Effects of Ca_o^{2+} and Sr^{2+} on cytosolic calcium (Ca_i^{2+}) concentration

We initially tested the effects of Ca_o^{2+} or Sr^{2+} alone on Ca_i^{2+} in CaR-transfected cells HEK293 cells using concentrations ranging from 0 to 9 mM (Fig. 1A). Increasing concentrations of either Ca_o^{2+} or Sr^{2+} produced transient followed by sustained increases in the cytosolic calcium concentration (Ca_i^{2+}) in CaR-transfected HEK293 cells. To compare the magnitudes of the transient increases in Ca_i^{2+} elicited by Ca_o^{2+} or Sr^{2+}, the data were expressed as the percentage of the maximal cumulative transient Ca_i^{2+} response observed with Ca_o^{2+}. Maximal responses were observed at 9 mM of both Ca_o^{2+} and Sr^{2+}, and the maximal cumulative response to Sr^{2+} was about 70% of that elicited by Ca_o^{2+}. Sustained Ca_i^{2+} levels were also larger with Ca_o^{2+} than with Sr^{2+}. The EC_{50} for Ca_o^{2+} was 3.4 mM, while that for Sr^{2+} was 4.4 mM in pooled data from three experiments. In contrast to their effects on CaR-transfected cells, neither Sr^{2+} nor Ca_o^{2+} had any effect on non-transfected HEK293 cells (not shown).

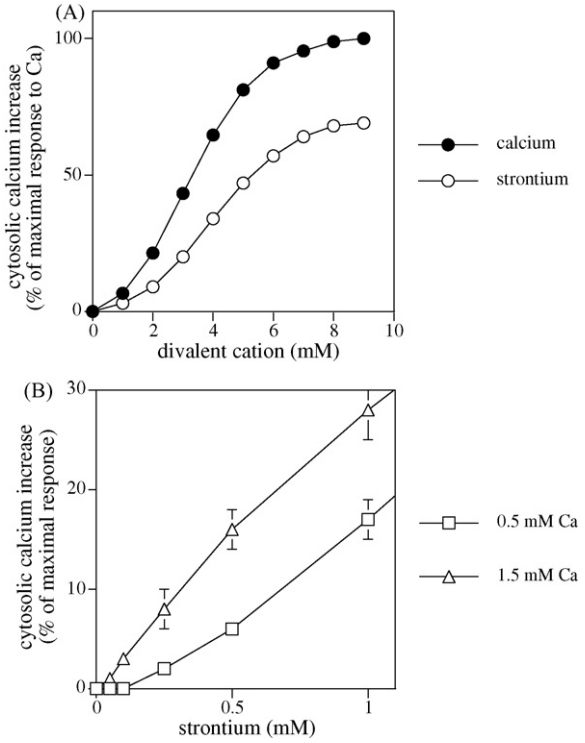

Fig. 1 – CaR-mediated Ca_i^{2+} responses in CaR-transfected HEK293 cells—effect of Ca_o^{2+} or Sr^{2+}. (A) The response to each cation is normalized to the maximal effect of calcium. Closed circles are for Ca_o^{2+} alone, and open circles for Sr^{2+} alone in the medium. The data points represent the mean ± S.E.M. from three experiments. In this and subsequent figures, if no error bar is shown, the S.E.M. is smaller than the size of the data point. (B) Data were plotted as the percentage of the maximal response for addition of Sr^{2+} in the presence of 0.5 mM and 1.5 mM Ca_o^{2+}. The cytosolic calcium responses to incremental additions of Sr^{2+} in the presence of 0.5 (open squares) or 1.5 mM Ca_o^{2+} (open triangles) were plotted against the Sr^{2+} concentration. Responses were normalized to the maximal response for each experiment. The data points represent the mean ± S.E.M. from 5–6 experiments. Changes in Ca_i^{2+} were monitored as ratio changes in the emission fluorescence of the calcium-sensitive dye, fura-2, when excited alternately by light at 340 and 380 nm, as described in Section 2. An increase in the ratio represents an increase in cytosolic calcium. Experiments were performed on populations of HEK 293 cells transiently transfected with the bovine CaR.

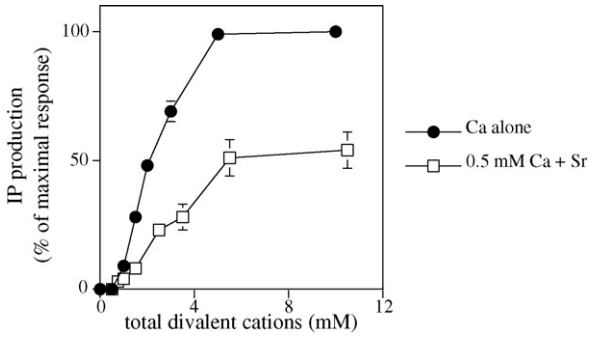

Fig. 2 – Effects of Sr^{2+} and Ca_o^{2+} on accumulation of IPs. Total IPs in CaR-transfected HEK293 cells incubated with varying concentrations of Sr^{2+} or Ca_o^{2+} were determined as described in Section 2. Experiments were carried out with Ca_o^{2+} alone (filled circle) or with 0.5 mM Ca_o^{2+} plus added Sr^{2+} (open square). IP production as a function of the total divalent cation concentration was expressed as the percentage of the maximal response seen at high Ca_o^{2+}. The data points represent the mean ± S.E.M. from three experiments performed in duplicate or quadruplicate.

The interactions between the effects of Sr^{2+} and Ca_o^{2+} on Ca_i^{2+} were evaluated by carrying out concentration-response experiments for Sr^{2+} (from 0.05 to 20 mM) in the presence of two different concentrations of Ca_o^{2+} (0.5 or 1.5 mM). In the presence of 0.5 mM Ca_o^{2+}, the maximal Ca_i^{2+} response was reached at Sr^{2+} concentrations between 5 and 10 mM (data not shown). The effects of Sr^{2+} on transient Ca_i^{2+} responses were then examined in the presence of 1.5 mM Ca_o^{2+}, corresponding to a physiological-free extracellular Ca^{2+}concentration. A response of 8 ± 2% of the maximal response was observed at 0.25 mM (Sr^{2+}) relative to that observed with 1.5 mM Ca_o^{2+} alone, which was defined as 0% for the purpose of this experiment (Fig. 1B). At 1 mM Sr^{2+}, substantial activation of Ca_i^{2+} responses was seen in the presence of both concentrations of Ca_o^{2+}, an effect that was not observed with Sr^{2+} alone (Fig. 1A). Thus, the cooperativity that occurs among CaR agonists in the activation of the receptor allows relatively low concentrations of secondary agonists (such as Sr^{2+}) to have pronounced effects on the CaR in the presence of physiological concentrations of its primary agonist, Ca_o^{2+}.

3.2. *Effects of Ca_o^{2+} and Sr^{2+} on accumulation of inositol phosphates*

We next examined the effects of Ca_o^{2+} and Sr^{2+} on the accumulation of total IPs in HEK293 cells transfected with the bovine CaR. Total IPs (e.g., IP, IP_2, IP_3 and IP_4) were used as an index of the activation of PLC. The results shown in Fig. 2 are expressed as the percentage of the maximal effect found with Ca_o^{2+} alone. Ca_o^{2+} by itself produced a dose-dependent increase in IPs, reaching a plateau between 5 and 10 mM Ca_o^{2+}, with an EC_{50} of 2.2 mM.

Sr^{2+} also elicited a concentration-dependent increase in total IPs in the presence of 0.5 mM Ca_o^{2+} with an EC_{50} of 2.7 mM. Under these conditions, the maximal effect observed was ~50% of that observed with Ca_o^{2+} alone. Addition of Sr^{2+} to 1.0 or 1.5 mM Ca_o^{2+} elicited smaller changes in IP production, when compared to Ca_o^{2+} alone (not shown), indicating that the apparent affinity and efficacy of Sr^{2+} were less than those of Ca_o^{2+}. However, submillimolar concentrations of Sr^{2+} did stimulate consistent, but small increases in IP production. Neither 5 mM Ca_o^{2+} nor 5 mM Sr^{2+} had any effect on total IPs in non-transfected cells (data not shown).

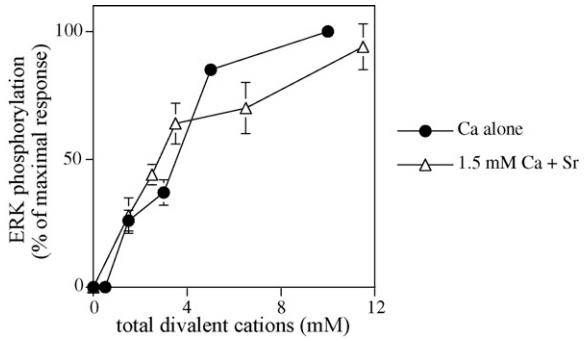

Fig. 3 – Effect of CaR activation by Ca_o^{2+} and Sr^{2+} on ERK1/2 activation. Effects of Ca_o^{2+} on ERK1/2 phosphorylation; the response is normalized to the maximal effect obtained with Ca_o^{2+}. Effects of varying concentrations of Sr^{2+} in the presence of 1.5 mM Ca_o^{2+}; the response is normalized to the maximal effect obtained with (Ca_o^{2+} + Sr^{2+}). Filled circles are for Ca_o^{2+}, and open triangles represent 1.5 mM Ca_o^{2+} plus various concentrations of added Sr^{2+}. ERK1/2 activity was assessed by quantitating the level of phosphorylated ERK1/2 protein using a phospho-specific MAPK antibody and Western blotting of cellular lysates as described in Section 2. The data points represent the mean ± S.E.M. from one experiment performed in triplicate.

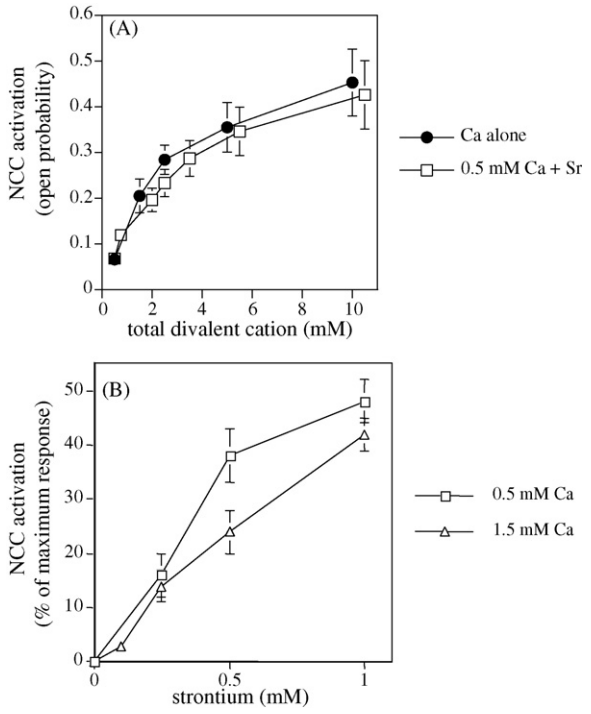

Fig. 4 – Effects of Ca_o^{2+} and Sr^{2+} on NCC activation in the presence of varying Ca_o^{2+} concentrations. (A) NCC activity was expressed as the percentage of the maximal response to high Ca_o^{2+} and as a function of the total divalent cation concentration. Filled circles are for Ca_o^{2+} alone, and open squares are for Sr^{2+} in the presence of 0.5 mM Ca_o^{2+}. (B) NCC activity at varying concentrations of Sr^{2+} was expressed as the percentage of the maximal response for each experiment plotted against the added Sr^{2+} concentration in the presence of 0.5 mM (open squares) or 1.5 mM Ca_o^{2+} (open triangles). NCC activity was recorded using the on-cell patch clamp technique on CaR-transfected HEK293 cells as described in Section 2. The data points represent the mean ± S.E.M. from four experiments.

3.3. *Effect of Ca_o^{2+} and Sr^{2+} on MAPK activity*

Previously, we have shown that activation of the CaR by elevated Ca^{2+} resulted in the phosphorylation of ERK1/2 in HEK-CaR cells but not in non-transfected HEK cells [26]. In HEK-CaR cells, the MEK–ERK pathway participates in the CaR-stimulated secretion of PTHrP [32]. Therefore, stimulation of phosphorylation of ERK1/2 in HEK-CaR cells was considered as a useful parameter to assess whether Sr^{2+} is a CaR agonist. Raising the level of Ca_o^{2+} substantially increased the level of phospho-ERK1/2 (Fig. 3). Maximal activation by Ca_o^{2+} was approached at 10 mM, and the EC_{50} for Ca_o^{2+} under these conditions was 3.6 mM. The effects of Sr^{2+} (from 1 to 15 mM) on MAPK activation were tested in the presence of 1.5 mM Ca_o^{2+}. The maximal activation induced by Sr^{2+} was observed at a concentration between 10 and 15 mM (not shown) and was similar to that of calcium (Fig. 3). We have previously shown that elevated levels of Ca_o^{2+} have no effect on the activity of MAPKs in non-transfected HEK cells [26].

3.4. *Effect of Ca_o^{2+} and Sr^{2+} on the activity of a non-selective cation channel*

Regulation of NCCs by the CaR has been shown in several cell types [27,33,34]. Previously, we showed that the CaR regulates an NCC in HEK-CaR cells [35]. Therefore, this was used as another CaR-stimulated readout in HEK-CaR to demonstrate that Sr^{2+} is a bona fide CaR agonist. In transfected HEK293 cells, Ca_o^{2+} produced a dose-dependent increase in the activity of the NCC (Fig. 4A). Under these experimental conditions, the open probability of the channel (the fraction of the time that the channel was in the open state) at 5 mM Ca^{2+} was 77% of the open probability at 10 mM Ca_o^{2+}, and the maximal observed activity was reached at a concentration of 10 mM Ca_o^{2+}. Higher concentrations could not be tested, because the cells tended to detach from the support. At 10 mM Ca_o^{2+}, there was about a seven-fold increase in the activity of the channel as assessed by the increase in its open state probability relative to that at 0.5 mM Ca_o^{2+}. The apparent affinity of Ca_o^{2+} for the CaR-mediated activation of the NCC was estimated by the EC_{50}, having a mean value of 2.2 mM. Ca_o^{2+} had only small, non-significant effects on the open state probability of the non-selective cation channel in non-transfected HEK293 cells (0.5 mM Ca_o^{2+}, 0.061 ± 0.011; 5 mM Ca_o^{2+}, 0.085 ± 0.063). However, the NCC was still available in the non-transfected cells, since addition of thapsigargin, which activates the channel by preventing uptake of calcium into the endoplasmic reticulum and thereby increasing Ca_i^{2+}, produced a robust increase in the open state probability of the

channel (from 0.061 ± 0.011 at 0.5 mM Ca_o^{2+} to 0.358 ± 0.063 with thapsigargin, 1 μM).

Sr^{2+} produced a dose-dependent increase in the activity of the channel in the presence of 0.5 mM Ca_o^{2+}, which was similar to that found with Ca_o^{2+} alone (Fig. 4A). In the presence of 0.5 mM and 1.5 mM Ca_o^{2+}, NCC activation could be observed with submillimolar concentrations of Sr^{2+} (Fig. 4B), as with the other transduction systems examined in this study. Because cells detached in the presence of high concentrations of divalent cations, compromising maintenance of the patch clamp seal, it was difficult to determine a precise value for the EC_{50}s of the two divalent cations. Both divalent cations appeared to have similar potencies as judged by their similar dose dependencies at 10 mM and below when the total divalent cation concentration was considered (Fig. 4A).

3.5. *Increasing concentrations of Sr^{2+} stimulate proliferation of calvarial OBs through the CaR*

We then examined the effects of strontium on the proliferation of rat primary calvarial osteoblasts in order to determine if the CaR could serve as a mediator for the previously documented anabolic action of Sr^{2+} on OBs [10]. Using rat primary calvarial OBs without any passaging, we tested whether the CaR-mediated high Sr^{2+}-induced proliferation, similar to the proliferative action of high Ca_o^{2+} that we have shown previously. BrdU incorporation was utilized as a parameter closely related to cell proliferation. As shown in Fig. 5, increasing concentrations of Sr^{2+} stimulated BrdU incorporation in BG-transfected cells, but the effect of Sr^{2+} was significantly attenuated in DNCaR transfected cells.

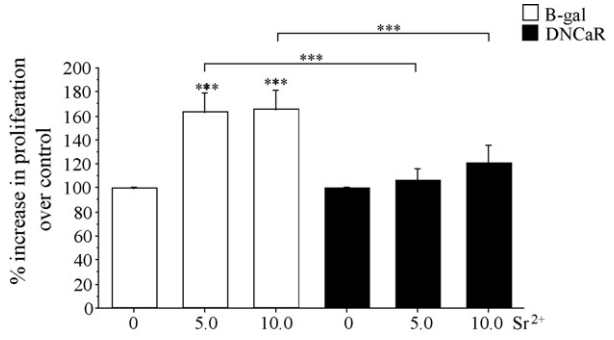

Fig. 5 – High Sr^{2+} stimulates proliferation of calvarial OBs via the CaR. Cells were plated directly in 96-well plates at 3–4 × 10^3/well following collagenase digestion. After 16 h, cells were infected with rAAV expressing either DNCaR or BG and cultured for 48 h before stimulation as described in Section 2. The cells were then starved for 4 h in serum-free medium containing 0.5 mM Ca_o^{2+} followed by addition of 0, 5 and 10 mM Sr^{2+} in serum-free medium and incubated for 16 h. Six wells were used for each Sr^{2+} concentration. After 16 h of incubation, cells were pulsed with BrdU for 3 h, and the color was developed as described in Section 2. Statistics were performed on raw data; $^{***}P < 0.001$ compared with control (0) or BG, $n = 6$ in two independent experiments.

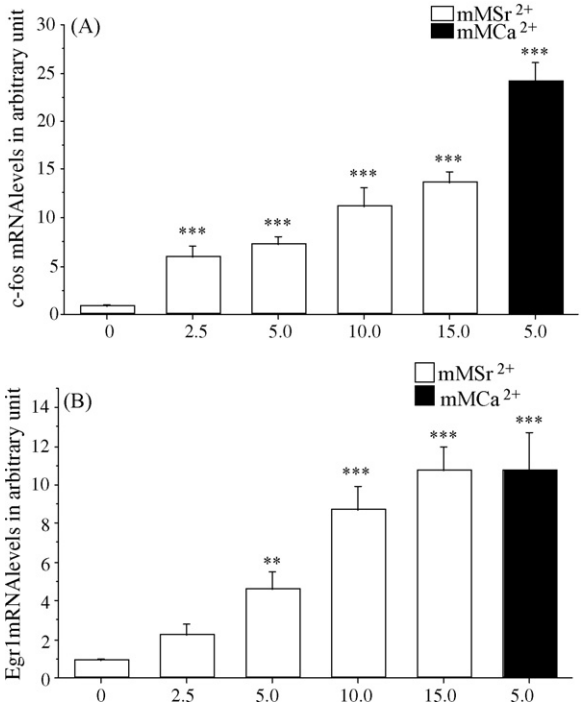

Fig. 6 – Sr^{2+} upregulates c-fos and egr-1 mRNAs in OBs via the CaR. Cells were plated directly in 24-well plates at 50–60 × 10^3/well following collagenase digestion. The cells were then starved for 4 h in serum-free medium followed by addition of increasing concentrations of Sr^{2+} in serum-free medium and incubation for 30 min. RNA was extracted by pooling two wells, and the levels of the transcripts for c-fos and egr-1 were assessed by real time PCR as described in Section 2. (**) $p < 0.01$, (***)$p < 0.001$ vs. control (0); one experiment performed in triplicate.

3.6. *Increasing concentrations of Sr^{2+} upregulate expression of c-fos and egr-1 in calvarial OBs via the CaR*

We have recently shown that elevated Ca_o^{2+} robustly induces c-fos and egr-1, known mitogenic protooncogenes, in OBs [35,36]. In order to test whether Sr^{2+} upregulates these mRNAs in OBs, we used QPCR determination of c-fos and egr-1 transcripts in cells treated with increasing concentrations of Sr^{2+} for 30 min, using 5 mM Ca_o^{2+} as a positive control. Fig. 6A and B shows that Sr^{2+} dose-dependently upregulated expression of transcripts for both c-fos and egr1, and that 5 mM Ca_o^{2+} is several-fold more effective than 5 mM Sr^{2+} in upregulating these two mRNAs in OBs.

Next, we tested whether the Sr^{2+}-evoked increase in the mRNAs for c-fos and egr-1 are mediated by the CaR by employing the same strategy of rAAV-mediated, high efficiency transfer of the dominant negative CaR into OB cells that was utilized above, again using the β-gal-containing vector as control. Fig. 7A shows that whereas Sr^{2+} induces c-fos mRNA in BG-transfected OBs, the effect was attenuated by approximately 60% in DNCaR-transfected cells. Similarly, Fig. 7B shows that the CaR also mediates the effect of Sr^{2+} in upregulating egr-1 mRNA in OBs, since cells transfected with

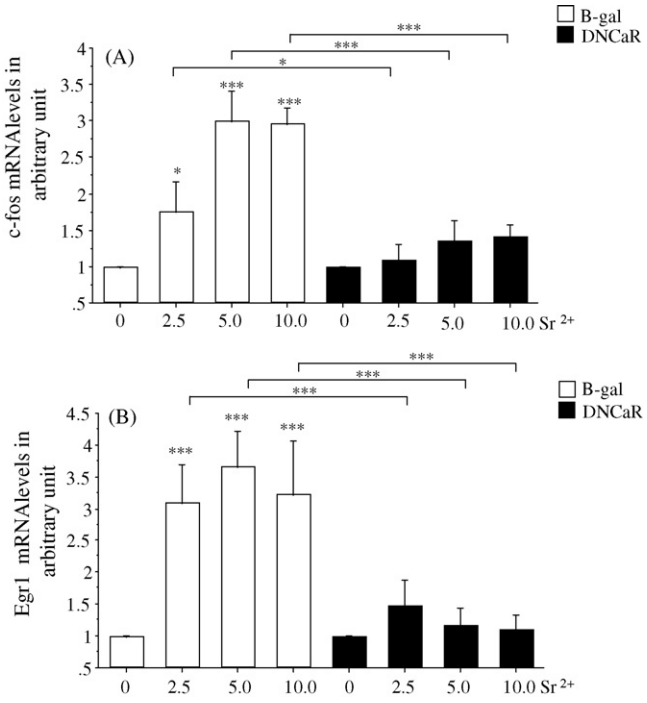

Fig. 7 – Sr^{2+} upregulates fos and egr-1 mRNAs in OBs via the CaR. Cells were plated directly in 24-well plates following collagenase digestion at 50–60 × 10^3/well. After 16 h, cells were infected with rAAV expressing either DNCaR or the vector control and cultured for 48 h before stimulation as described in Section 2. The cells were then starved for 4 h in serum-free medium followed by addition of increasing concentrations of Sr^{2+} in serum-free medium and incubation for 30 min. Extracted RNA was pooled from two wells, and the levels of the transcripts for c-fos and egr-1 were assessed by real time PCR as described in Section 2. (*) $p < 0.05$, (***)$p < 0.001$ compared with control (0) or BG; one experiment performed in triplicate.

DNCaR exhibit ~65% decrease in expression of egr-1 in response to elevated Sr^{2+} compared with BG-infected cells. These data suggest that Sr^{2+}-stimulated upregulation of c-fos and egr-1 mRNAs are CaR-mediated in rat primary calvarial OBs.

4. Discussion

We hypothesized that strontium ranelate enhances bone formation due to the effects of Sr^{2+} on the calcium-sensing receptor. In the first part of the study, our results indicate that Sr^{2+}, like Ca_o^{2+}, is an agonist of the CaR since Sr^{2+} concentration-dependently stimulated transient and sustained increases in Ca_i^{2+}, accumulation of IPs, ERK1/2 phosphorylation and the activity of a Ca_o^{2+}-permeable NCC in CaR-transfected (HEK-293) but not in non-transfected cells [26,27,31].

The potency of Sr^{2+} as an agonist of the CaR varied depending on the biological response tested. For example, the maximal transient increase in Ca_i^{2+} obtained with Sr^{2+} was 70% of that produced by Ca_o^{2+}, suggesting greater mobilization of Ca_i^{2+} stores by Ca_o^{2+} as compared with Sr^{2+}. Indeed Sr^{2+} appeared to have a lower affinity than Ca_o^{2+} for CaR-mediated IP production (EC_{50} of 2.7 versus 2.2 mM), and Sr^{2+} also produced a smaller maximal response.

The reasons for the reduced stimulation of IP production by Sr^{2+} compared to Ca_o^{2+} could be several-fold. First, the extent to which various PLC isoforms are stimulated by Sr^{2+} and Ca^{2+} following CaR activation could vary [37]. Indeed, data from our laboratory indicate that elevated Ca_o^{2+} stimulates a highly Ca^{2+}-dependent PLC isoform in HEK-CaR which generates substantially more IP compared with other polycationic CaR agonists, such as neomycin, spermine and gadolinium (M. Bai and E.M Brown, unpublished observations). Second, Sr^{2+} *per se* may not activate PLC with the same affinity and efficacy as does Ca^{2+}. Third, different channel properties of the HEK-CaR cells compared to other cells in the conduction of cations could explain variable Ca^{2+} and Sr^{2+} response in eliciting sustained Ca^{2+} influx. Sr^{2+} typically enters most cells via voltage-dependent and -independent Ca^{2+}-permeable channels, and HEK293 cells possess calcium channels that have a low permeability for Sr^{2+}. In fact, HEK293 cells have a prominent non-selective Ca^{2+}-permeable channel that does not conduct Sr^{2+} [38], and CaR activation results in the opening of a NCC in HEK-CaR cells, which will lead to Ca^{2+} influx [27]. Similar observations were made in studies examining the effects of Sr^{2+} and Ca_o^{2+} on IP accumulation in AtT20 cells, which express an endogenous CaR and in CaR-transfected CHO cells [23,24]. They likewise found that Sr^{2+} had a lower apparent affinity than Ca^{2+}, but Ca_o^{2+} and Sr^{2+} stimulated IP accumulation to a similar maximal extent.

Both Sr^{2+} and Ca_o^{2+} had comparable effects in stimulating phosphorylation of ERK1/2 and NCC activation. In this regard, Sr^{2+} appears to be an effective surrogate agonist of the CaR. We have previously shown that elevated Ca_o^{2+} stimulates phosphorylation of ERK1/2 in CaR-transfected but not in non-transfected cells via a pertussis toxin-sensitive PKC pathway [26]. Thus, Sr^{2+}, by activating the CaR, could exert its biological actions through at least two pathways: (a) activation of PLC [24,31], and (b) stimulation of the MEK–ERK pathway [26]. Small increases in Sr^{2+} elicit measurable increases in the open state probability of the NCC, and there is a steep dose-response relationship that is similar for the Ca_i^{2+} responses to Ca_o^{2+} and Sr^{2+}. Therefore, NCC activation is equally sensitive to both Ca_o^{2+} and Sr^{2+}.

Although low concentrations of Sr^{2+} (0.25–0.5 mM) were able to activate the receptor in the presence of a near physiological Ca_o^{2+} concentration of 1.5 mM, these concentrations are above that achieved in blood (~0.1 mM) during therapeutic treatment with strontium ranelate (2 g/d) in postmenopausal women [2]. Therefore, therapeutic Sr^{2+} concentrations in plasma should have little activity on peripheral tissues expressing the CaR, such as parathyroid and kidney [for review 15]. However, at sites where the levels of either Sr^{2+} or Ca_o^{2+} might be higher than those in the blood, such as in bone [39], strontium could potentially modulate the activity of the CaR in cooperation with Ca_o^{2+}.

The second aim of the present study was to investigate the effects of Sr^{2+} on the proliferation of POB. Sr^{2+} has been shown to enhance both pre-OB proliferation and bone formation in

cell and calvarial culture [10] and to have bone anabolic effects *in vivo* [1–3]. Recently, we [21] and others [22] have shown that high Ca_o^{2+}, acting via the CaR, stimulates the proliferation of rat calvarial POB. Here, we show that increasing concentrations of Sr^{2+} likewise promote POB proliferation and that this effect is attenuated following transduction of a dominant negative CaR (R186Q) via rAAV. Therefore, we conclude that one of the molecular targets of Sr^{2+} in mediating POB proliferation is the cloned CaR that mediates the actions of calcium on parathyroid and kidney [13,15]. However, we cannot rule out the theoretical possibility that the dominant negative bovine CaR heterodimerizes and inactivates a structurally related cation-sensing receptor, GPRC6A, that is expressed in OBs [16].

Finally, this study investigated the effect of Sr^{2+}-induced CaR activation on expression of c-fos and egr-1. C-fos has been reported to be involved in the regulation of OB proliferation, differentiation and ultimately bone formation [21,35,36]. Elevated levels of c-fos are associated with regions of fetal bone having the highest growth potential [40], and c-fos is regulated *in vitro* and *in vivo* by PTH (1–34) [41,42]. Egr-1, a nuclear Zn^{2+} finger protein and transcriptional regulator, has been shown to be co-regulated with c-fos in developing mouse bone and shows an overlapping pattern of expression at the ossification site in mesenchymally derived cells [35,43]. Recently, we have shown that Ca_o^{2+} rapidly activated c-fos and egr-1 in OBs [21]. However, whether those effects were CaR-mediated was not determined. Here, we show that, similar to the effects of Ca_o^{2+}, Sr^{2+} stimulated mRNA levels of c-fos and egr-1 mRNAs in POBs and dominant negative CaR transduction attenuated this effect. These data suggest that CaR mediates the stimulatory effects of Sr^{2+} on mRNA levels of c-fos and egr-1.

Viral transduction in some cells is known to activate c-fos and egr-1 [44]. In our hands, viral transduction with the rAAV utilized here resulted in upregulation of c-fos and egr1 mRNAs thereby reducing the fold increase achieved by increasing concentrations of Sr^{2+} alone. Nevertheless, the remaining stimulation of c-fos and egr-1 above the baseline was robust, and the dominant negative CaR significantly attenuated the Sr^{2+}-stimulated increase in c-fos and egr-1 mRNAs. It remains to be ascertained whether the upregulation of c-fos and egr-1 resulting from the activation of the CaR by elevated Sr^{2+} mediate the proliferative effect of Sr^{2+} on OBs. In addition, while others have shown calcium-evoked increases in the expression of various gene products reflecting OB differentiation, we did not observe effects other than on proliferation, c-fos and egr-1 in the rat POB utilized here, perhaps as a result of differences in the OB cell types studied.

In summary, the results obtained from both heterologous and endogenously expressing systems clearly show that Sr^{2+} is an agonist of the CaR and that this receptor is involved in mediating several biological effects of strontium raneleate on POBs. In HEK293 cells, Sr^{2+}, acting via the CaR, stimulates four biological responses e.g., elevations of Ca_i^{2+}, IPs, stimulating ERK1/2 pathway and activation of the NCC. Sr^{2+} may be relatively more efficient in activating ERK1/2 and NCC than in stimulating IP accumulation or Ca_i^{2+} responses, suggesting that Ca^{2+} and Sr^{2+} have differential effects on specific cellular processes. As a consequence, Sr^{2+} may be a more effective CaR agonist in some cells than others, and its effectiveness may be dependent on the transduction pathways utilized by any given cell for modulating its cellular responses. In POBs, strontium ranelate via the CaR increases cell proliferation and the expression of associated genes. These effects are consistent with the known anabolic action of strontium ranelate in OBs.

Conflicts of interest statement

None declared.

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