The Calcium-sensing Receptor Is Involved in Strontium Ranelate-induced Osteoclast Apoptosis
NEW INSIGHTS INTO THE ASSOCIATED SIGNALING PATHWAYS

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Strontium ranelate exerts both an anti-catabolic and an anabolic effect on bone cells. To further investigate the molecular mechanism whereby strontium ranelate inhibits bone resorption, we focused our attention on the effects of strontium ranelate on osteoclast apoptosis and on the underlying mechanism(s). Using primary mature rabbit osteoclasts, we demonstrated that strontium (Sr\(^{2+}\)) dose-dependently stimulates the apoptosis of mature osteoclasts. As shown previously for calcium (Ca\(^{2+}\)), the Sr\(^{2+}\)-induced effect on mature osteoclasts is mediated by the Ca\(^{2+}\)-sensing receptor, CaR, which in turn stimulates a phospholipase C-dependent signaling pathway and nuclear translocation of NF-κB. Unlike Ca\(^{2+}\), however, Sr\(^{2+}\)-induced osteoclast apoptosis was shown to depend on PKCβII activation and to be independent of inositol 1,4,5-trisphosphate action. As a consequence of these differences in their intracellular signaling pathways, Sr\(^{2+}\) and Ca\(^{2+}\) in combination were shown to exert a greater effect on mature osteoclast apoptosis than did either divalent cation by itself. Altogether, our results show that Sr\(^{2+}\) acts through the CaR and induces osteoclast apoptosis through a signaling pathway similar to but different in certain respects from that of Ca\(^{2+}\). This difference in the respective signaling cascades enables Sr\(^{2+}\) to potentiate Ca\(^{2+}\)-induced osteoclast apoptosis and vice versa. In this manner, it is conceivable that Sr\(^{2+}\) and Ca\(^{2+}\) act together to inhibit bone resorption in strontium ranelate-treated patients.

In the past decade, several effective compounds have been developed for the treatment of osteoporosis. Vertebral and nonvertebral fractures are decreased 30–65% by administration of anti-catabolic agents (such as raloxifene, alendronate, and risedronate) or anabolic agents (such as fragments of parathyroid hormone).

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Strontium ranelate, which is composed of an organic moiety and two atoms of stable strontium (Sr\(^{2+}\)), is a novel effective compound in the treatment of postmenopausal osteoporosis, reducing the risk of vertebral and hip fractures (1–3). In vitro strontium ranelate acts on both osteoblasts and osteoclasts (4–9). Similarly, in vivo, in contrast to other agents designed for treating osteoporosis, strontium ranelate uncouples bone turnover in favor of bone formation by both stimulating bone formation and decreasing bone resorption (10–15). Thus, in ovariectomized (OVX) rats, strontium ranelate prevents the associated bone loss, as indicated by histomorphometric indices of bone resorption (osteoclast surfaces), while maintaining indices of bone formation (osteoblast surface, bone formation rate, and alkaline phosphatase) at a high level (16). Because of this dual mechanism of action, strontium ranelate prevents the decrease in ultimate load to failure of bone studied in vitro by preventing the deterioration of bone microarchitecture and intrinsic bone tissue quality, as observed in 6-month-old OVX Sprague-Dawley rats treated for 52 weeks with strontium ranelate (125, 250, or 625 mg/kg/day) (17). Activation of the CaR has been suggested to be among the factors contributing to this mechanism (18). However, the precise cellular events through which Sr\(^{2+}\) acts as an uncoupling agent are still under investigation.

We recently established that calcium (Ca\(^{2+}\))-sensing through the Ca\(^{2+}\)-sensing receptor (CaR)\(^{3}\) leads to signaling via IP\(_{3}\), which stimulates nuclear translocation of NF-κB. This, in turn, induces apoptosis of mature osteoclasts (19), which serves as a key step in the regulation of overall osteoclast activity and thereby in the process of bone resorption (20). Because Sr\(^{2+}\) closely resembles Ca\(^{2+}\) in its atomic and ionic properties and because they are both agonists of the CaR (8, 21), we hypothesized that Sr\(^{2+}\) may regulate mature osteoclast apoptosis directly via the CaR. In the present studies, investigating the effect of Sr\(^{2+}\) on mature rabbit osteoclasts, we established that...
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it promotes osteoclast apoptosis in a dose-dependent manner. Moreover, we demonstrated that Sr$_{2}^{+}$-induced effects were mediated through activation of the CaR followed by stimulation of phospholipase C (PLC) and nuclear translocation of NF-$\kappa$B. The effects of Sr$_{2}^{+}$ were not, however, mediated by IP$_{3}$-dependent signaling per se but by activation of diacylglycerol (DAG)-PKC$\beta$II signaling, in contrast to Ca$_{2}^{+}$-induced apoptosis. We further demonstrated that Sr$_{2}^{+}$ potentiates the effects of Ca$_{2}^{+}$ on all of the biological activities that we assessed.

**EXPERIMENTAL PROCEDURES**

**Animals and Reagents**—Ten-day-old New Zealand White rabbits were purchased from Elevage Scientifique des Dombes (Chatillon/Chalaronne, France). Because of the limited solubility of strontium ranelate in the culture medium, strontium chloride (Sigma) mixed with sodium ranelate (Technologie Servier, Orléans, France) was used to test concentrations of strontium ranelate up to 30 mM (expressed in mM Sr$_{2}^{+}$). A 1/100 ratio between ranelic acid and Sr$_{2}^{+}$ was used to mimic the therapeutic circulating ratio of these two substances observed in patients treated with strontium ranelate. At concentrations as high as 0.3 mM, the ranelate moiety does not change either the effect of Sr$_{2}^{+}$ on osteoclast apoptosis or the osmolarity of the culture medium (data not shown). Tocris Cookson Ltd. (Bristol, UK) provided inhibitors of PLC (U73122 and U73343). Blockers of IP$_{3}$-dependent intracellular signaling ([2-aminoethoxydiphenyl borate (2-APB) and 1-[β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1H-imidazole (SKF-96365)); DAG analogues 1-oleoyl-2-acetyl-sn-glycerol (OAG); PKC agonists phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), and 4-α-phorbol; PKC inhibitors (calphostin C, 7-hydroxy-stauroporine, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)-pyrrolo(3,4-c)-carbazole (Gö-6976), bisindolylmaleimide XI (Ro-32-0432), and 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF 109203X)); and an NF-$\kappa$B inhibitor (Ro106-9920) were obtained from Calbiochem. GF 109203X was used to inhibit both Ca$^{2+}$-dependent (α, β, βII, and γ) and Ca$^{2+}$-independent (δ and ε) PKC isozymes. Gō-6976 selectively inhibits the Ca$^{2+}$-dependent PKCα and PKCβ1 isozymes without affecting the activities of the Ca$^{2+}$-independent PKCδ, ε, and ζ isozymes; and Ro-32-0432 selectively inhibits Ca$^{2+}$-dependent PKCα and PKCβ1 isoforms. Based on preliminary data assessing the concentrations of these agents that could be used without causing any deleterious effects on mature osteoclasts, we determined that the optimal concentrations of GF 109203X, Gō-6976, and Ro-32-0432 were 100, 1, and 0.1 nM, respectively (data not shown). Collagenase/dispose and fetal calf serum (FCS) were obtained, respectively, from Roche and Dominique Dutscher S.A. (Brumath, France). Culture plates were purchased from Corning.

**Osteoclast Isolation and Culture**—Mature osteoclasts were isolated from the long bones of rabbits according to the procedure described by Foged et al. (22), with slight modifications. Briefly, rabbit long bones were dissected and minced with scissors in α-MEM supplemented with 10% heat-inactivated FCS. The cells were then dissociated from the bone fragments by vigorous vortexing and collected by centrifugation (4 min, 500 rpm) prior to being seeded on 24-well plates or on 12-mm glass coverslips.

Purified osteoclasts, which were used to directly assess the effect of Sr$_{2}^{+}$ on apoptosis of mature osteoclasts, were obtained by removing osteoblasts as well as stromal cells from the wells using a solution of 0.01% collagenase/dispose prepared in phosphate-buffered saline. Purified osteoclasts were then incubated in the medium for 2 h. Next, the cells were cultured for 48 h in α-MEM supplemented with 1% FCS containing various concentrations of tested substances (Sr$_{2}^{+}$ and/or Ca$_{2}^{+}$) and pharmacological agonists/antagonists. Tartrate-resistant acid phosphatase (TRAP)-positive cells were assessed by staining with a leukocyte acid phosphatase kit (Sigma 387-A). In all cases, cell purity was assessed using TRAP staining and was close to 99%. Only TRAP-positive cells that contained three or more nuclei were considered to be mature osteoclasts. As indicated hereafter, prior to being placed in culture, some plated cells were transfected with the DN-CaR or small interfering RNA (siRNA) to assess the role played by the CaR and PKCs in Sr$_{2}^{+}$-induced effects.

To assess the nuclear translocation of NF-$\kappa$B, rabbit bone cells were submitted to an additional step prior to being seeded on 12-mm glass coverslips. As first described by Collin-Osdoby et al. (23), the bone cell pellet was resuspended in 15 ml of serum-free medium and was carefully added to the top of a 70–40% FCS gradient. The preparation was then left undisturbed for 30 min to allow the larger multinucleated osteoclasts to settle under unit gravity and penetrate the FCS layers. Prior to being seeded on glass coverslips, the bottom 15 ml of the gradient, which contained predominantly mature osteoclasts, was harvested, centrifuged for 5 min at 700 rpm, and resuspended in α-MEM supplemented with 10% FCS.

**Detection and Quantification of Osteoclast Apoptosis**—As previously described by Kameda et al. (24), after treatment with reagents, cells were fixed with 3.7% formaldehyde for 10 min and stained with 0.2 μM Hoechst 33258 for 10 min. Cells were examined under a fluorescence microscope (Olympus BH2) for determination of morphological changes of the chomatin as described previously (25, 26). At least 100 multinucleated cells were scored per well in order to assess the prevalence of apoptotic changes in the chromatin, and the extent of apoptosis of mature osteoclasts was expressed as the percentage of apoptotic osteoclasts relative to the total number of osteoclasts. Cell-permeable peptide inhibitors of the caspase cascade (i.e. benzoyloxycarbonyl-Val-Ala-Asp (Ome)-fluoromethyl ketone (Z-VAD-fmk) and benzoyloxycarbonyl-Leu-Glu-His-Asp (Ome)-fluoromethyl ketone (Z-LEHD-fmk)), used to assess the role of caspases in Sr$_{2}^{+}$ ranelate-induced osteoclast apoptosis, were obtained from Calbiochem. Apoptosis was confirmed by annexin V-FITC staining using an annexin V-FITC fluorescence microscopy kit (BD Biosciences).

**NF-$\kappa$B Localization by Immunofluorescence**—Rabbit osteoclasts seeded on glass coverslips were incubated with various test substances in osteoclast culture medium at 37 °C. Treatments were started at various times prior to fixation with 3.7% formaldehyde (5 min) as described in subsequent sections. Cells were washed twice with phosphate-buffered saline and incu-
bated for 10 min in a 0.5% Triton X-100-phosphate-buffered saline solution. Osteoclasts were then incubated overnight at 4 °C with a mouse anti-p65 primary antibody (Santa Cruz Biotechnology; sc-8008) and subsequently incubated for 1 h at room temperature with the AlexaFluor-488-conjugated goat anti-mouse IgG (H+L) secondary antibody. Coverslips were finally mounted on slides using VECTASHIELD® before examination using a confocal microscope. The entire osteoclast population was examined (usually 100–200 osteoclasts/coverslip).

Gene Delivery by Recombinant Adeno-associated Virus—High efficiency gene transfer into mature rabbit osteoclasts was accomplished using a recombinant adeno-associated virus-based method. A bovine CaR sequence with a naturally occurring dominant-negative mutation, R186Q (DN-CaR) (27), or the same vector encoding β-galactosidase cDNA (as a control for nonspecific effects of viral infection) were placed under the control of a cytomegalovirus immediate-early (CMV-IE) promoter element and packaged in the same vector as described previously (28). Prior to being exposed to the virus, mature rabbit osteoclasts were cultured overnight in α-MEM supplemented with 10% FCS. Cells were then washed once with serum-free α-MEM, and about 1,000 viral particles/cell were used to infect each well (as optimized by pilot studies). Cells were incubated for 90 min in serum-free medium at 37 °C in a cell culture incubator. Then, equal volumes of α-MEM containing 20% serum were added to the cells to achieve a final serum concentration of 10%. The cells were finally cultured for 36 h prior to being used for the experiments described in subsequent sections. On each coverslip, at least 80% of the osteoclasts appeared to be Alexafluor®-488-positive (data not shown).

Small Interfering RNA—siRNA design and synthesis were carried out in collaboration with Eurogentec (Liege, Belgium) based on the reference sequences NM000388 (human cDNA) and X04796, X04795, and X04793 (rabbit cDNAs). CaR siRNA were designed in regions of the cDNA that are homologous with the rabbit sequence (29); sequences will be provided upon request. siRNA transfections were carried out in triplicate on purified mature rabbit osteoclasts. To transfect mature rabbit osteoclasts, 0.2 μl of siRNA (100 μm) was diluted into 50 μl of Opti-MEM and added to each well. Next, 2.5 μl of NeoFx (Ambion, Inc.) was diluted into 50 μl of Opti-MEM for each sample and incubated for 10 min at room temperature, and then 50 μl of diluted transfection mixture was added. Finally the mixture (100 μl) was added to wells that contained purified osteoclasts. Cells and inhibitors were incubated for an additional 90 min at 37 °C with 5% CO₂. Then a volume of Opti-MEM containing 20% serum was added to the cells to achieve a final serum concentration of 10%. The cells were finally cultured for 36 h prior to being used for the experiments described in subsequent sections. As negative controls, parallel experiments were carried out using scrambled siRNAs, which did not match the sequences of any mammalian mRNAs (Ambion). For each targeted mRNA, i.e. CaR and PKC (α, βI, and βII), three siRNA were designed. Some of them were Alexafluor®-488-conjugated to visualize the transfection efficiency. On each coverslip at least 70% of the osteoclasts appeared to be Alexafluor®-488-positive (data not shown). In all of the transfected osteoclasts, the siRNA-Alexafluor®-488 complexes were located in the cytoplasm, where they are expected to exert their biological activities. The small number of mature osteoclasts available for these studies and their rabbit origin makes the extent of knockdown for PKCα, -βI, and -βII proteins difficult to assess. Therefore, depletion of endogenous mRNAs encoding CaR and PKCs (α, βI, and βII) by siRNA was confirmed by real-time PCR, and the respective transcripts were knocked down 85, 80, 75, and 85%.

Statistical Analysis—Results are expressed as the mean ± S.E. The statistical differences among groups were evaluated using the Kruskal-Wallis test. The Mann-Whitney U test was then used to identify differences between the groups when the Kruskal-Wallis test indicated a significant difference (p < 0.05).

RESULTS

Sr²⁺ Induces Apoptosis of Mature Osteoclasts in a Caspase-dependent Manner—Apoptosis of mature rabbit osteoclasts was detected and quantified by visualizing chromatin condensation of nuclei in apoptotic cells (Fig. 1A). As we had observed previously with CaR⁺⁺ (19, 25), increasing Sr²⁺⁺ promoted, in a dose-dependent manner, osteoclast apoptosis (figure 1B). After 48 h of culture in a medium containing 30 mM Sr²⁺⁺, 38 ± 1.5% of the osteoclasts were apoptotic, whereas in control cultures 14% ± 1 were apoptotic (p < 0.001). Significant differences were observed at Sr²⁺⁺ concentrations of 9 mM and higher (p < 0.05). Peptides inhibiting caspases 3 and 9, Z-VAD-fmk and Z-LEHD-fmk, respectively, significantly and dose-dependently inhibited Sr²⁺⁺-induced apoptosis of osteoclasts (Sr²⁺⁺ = 25 mM) (Fig. 1, C and D). Osteoclast apoptosis was confirmed through annexin V immunofluorescence (Fig. 1, E and F). The data show that untreated cells were negative for annexin V-FITC staining, whereas positive cells were seen following Sr²⁺⁺ (25 mM) treatment.

Through Stimulation of the CaR, Sr²⁺⁺ Induces Mature Osteoclast Apoptosis in an IP₃-independent but PLC-dependent Manner—DN-CaR transfection of mature rabbit osteoclasts partially but significantly inhibited Sr²⁺⁺-induced osteoclast apoptosis. DN-CaR-transfected osteoclast apoptosis was reduced by about 36% compared with that observed in β-galactosidase-transfected cells when transfected cells were cultured for 48 h in the presence of 20 mM Sr²⁺⁺ (Fig. 2A, p < 0.01). Notably, because wild type CaR homodimers would be unaffected by the DN-CaR or because of non-CaR action(s) of the Sr²⁺⁺, we were unable to completely eliminate Sr²⁺⁺-induced osteoclast apoptosis using the DN construct. To confirm the importance of a G protein-coupled receptor-based mechanism in the Sr²⁺⁺-induced osteoclast apoptosis, we investigated the roles of IP₃ signaling using specific pharmacological inhibitors (U73122 (PLC inhibitor) and SKF-96365 (IP₃ signaling pathway inhibitor)). As shown in Fig. 2B, U73122 (10 μM) abolished Sr²⁺⁺ (25 mM)-induced osteoclast apoptosis, whereas SKF-96365 (10 μM) did not. As we had shown previously that SKF-96365 is able to significantly inhibit CaR⁺⁺-induced osteoclast apoptosis utilizing the same cellular model of mature rabbit osteoclasts and the same concentration of the compound (19), we considered that SKF-96365...
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**FIGURE 1.** Sr\(^{2+}\) induces apoptosis of rabbit mature osteoclasts. Morphological changes of the nuclei were visualized by staining the cells with 0.2 mM Hoechst 33258. A, fluorescence micrograph (magnification ×200) showing both normal (arrowheads) and apoptotic (arrows) osteoclasts. B, increasing doses of Sr\(^{2+}\), ranging from 0 to 30 mM, dose-dependently stimulate apoptosis of mature osteoclasts. Data are expressed as means ± S.E. of three independent experiments (6 wells/condition were counted for each experiment). *, p < 0.05; **, p < 0.01; ***., p < 0.001; compared with controls. C and D show the effects of Z-VAD-fmk (caspase 3 inhibitory peptide) or Z-LEHD-fmk (caspase 9 inhibitory peptide) on Sr\(^{2+}\)-induced apoptosis of rabbit osteoclasts. Various concentrations of Z-VAD-fmk and Z-LEHD-fmk were introduced, in the absence or presence of Sr\(^{2+}\), for 48 h of culture. Data are expressed as means ± S.E. of two independent experiments. *, p < 0.05, and ***, p < 0.001; compared with cultures treated with Sr\(^{2+}\) (25 mM) alone. TRAP-positive osteoclasts were left untreated (E) or induced to undergo apoptosis with Sr\(^{2+}\) (25 mM) for 24 h and stained with annexin V-FITC according to the manufacturer’s instructions (F). E’ and F’ represent light micrographic images of E and F, respectively. Mature osteoclasts were stained (purple) for TRAP activity. The cells showing visible annexin V staining (arrows) were considered as apoptotic cells.

was biologically active under the conditions used here and that the IP\(_3\) signaling pathway was not implicated in Sr\(^{2+}\)-induced apoptosis of mature osteoclasts. Notably, U73343 (10 \(\mu\)M), a less potent pharmacological inhibitor of PLC, was without effect on Sr\(^{2+}\)-induced osteoclast apoptosis (data not shown).

Sr\(^{2+}\), but Not Ca\(^{2+}\), Stimulates Osteoclast Apoptosis in a PKC-dependent Manner—Activation of PLC leads to the breakdown of phosphatidylinositol 4,5-biphosphate (PIP\(_2\)) and the generation of two second messengers, IP\(_3\) and DAG. Based on the lack of effect of the inhibitors of the IP\(_3\) signaling pathway, we hypothesized that DAG production and the resultant PKC activation may play major roles in Sr\(^{2+}\)-induced osteoclast apoptosis. However, the role of PKC signaling in the regulation of osteoclast apoptosis has not yet been established. Therefore, we first assessed the roles played by DAG and the subsequent activation of PKC in the regulation of apoptotic cell death of osteoclasts using substances known to stimulate PKC signaling, namely a DAG analogue (OAG) and phorbol esters (PMA or PDBu). OAG induced, in a dose-dependent manner, osteoclast apoptosis. After 48 h of culture in the presence of 100 \(\mu\)M OAG, ~80% of the osteoclasts were apoptotic (Fig. 3A). Similar results were obtained when mature osteoclasts were cultured in the presence of PMA or PDBu (Fig. 3, B and C), which stimulated mature osteoclast apoptosis by 80 and 95% when cultured, respectively, in the presence of PMA (10 \(\mu\)M) and PDBu (1 \(\mu\)M). At these concentrations, an inactive phorbol ester, 4 α-phorbol, did not induce any apoptosis of osteoclasts (data not shown). Subsequently, the cells were cultured for 48 h in the presence of calphostin C and 7-hydroxy-staurosporine, two well known pan-PKC antagonists. Under these conditions, Sr\(^{2+}\)-induced osteoclast apoptosis was significantly reduced by 35 and 32%, respectively, compared with the rate of apoptosis observed in cultures treated with Sr\(^{2+}\) (25 mM) alone (Fig. 4A). Note that staurosporine (0.1 \(\mu\)M) and calphostin C (1 \(\mu\)M) failed to reduce Ca\(^{2+}\)-induced osteoclast apoptosis, indicating that Ca\(^{2+}\) stimulates osteoclast apoptosis in a PKC-independent manner (Fig. 4B). To further investigate the role of PKC isozymes in Sr\(^{2+}\)-induced osteoclast apoptosis, we used more specific PKC inhibitors. As shown in Fig. 4C, GF 109203X (100 \(\mu\)M), G6-6976 (1 \(\mu\)M) and Ro-32-0432 (0.1 \(\mu\)M) substantially and significantly reduced Sr\(^{2+}\)-induced osteoclast apoptosis. Based on these data, siRNAs specific for PKCs α, βI, and βII were transfected into mature rabbit osteoclasts for 36 h followed by the addition of Sr\(^{2+}\) (25 mM) for 48 h. In these culture conditions, only silencing of the PKCβII gene inhibited Sr\(^{2+}\)-induced osteoclast apoptosis (Fig. 4D). For each targeted PKC (α, βI, and βII)), three siRNAs were transfected and gave comparable results (data not shown). Presumably because of the additional 36 h needed to obtain efficient knockdown of the genes of interest, transfection of siRNA with scrambled sequences increased both basal and Sr\(^{2+}\)-induced osteoclast apoptosis from 13 ± 1% and 26 ± 2% (Fig. 4A) to 20 ± 2% and 37 ± 6%, respectively. Thus, Sr\(^{2+}\) (25 mM) still significantly stimulated apoptosis of mature osteoclasts compared with untreated cells (p < 0.05). In this regard, the Sr\(^{2+}\)-induced stimulation of apoptosis persists (~15% greater apoptosis), albeit with a higher base line, except in the case of mature rabbit osteoclasts transfected with PKCβII siRNAs.

Sr\(^{2+}\), through the Stimulation of PKCβII and NF-κB, Potentiates the Effects Exerted by Ca\(^{2+}\) while Acting on the Same
Receptor, the CaR—Because of the role played by NF-κB in Ca\(^{2+}\)-induced osteoclast apoptosis (19), we speculated that NF-κB activation might play a key role as a mediator of Sr\(^{2+}\)-induced osteoclast apoptosis. As shown in Fig. 5D, when osteoclasts were cultured for up to 12 h with Sr\(^{2+}\) (25 mM), nuclear translocation of NF-κB was transient and appeared to be as rapid as during RANKL-induced nuclear translocation of NF-κB (Fig. 5C). Maximum activation was observed at 30 min, when 49 ± 2% of the osteoclasts exhibited nuclear translocation of NF-κB (p < 0.001 compared with untreated cells). The nuclear translocation of NF-κB then decreased before a second phase of stimulation occurred, more sustained in duration, during which NF-κB nuclear translocation reached ~50–55%. Transfection of the cells with the DN-CaR dramatically reduced Sr\(^{2+}\)-induced NF-κB activation showing that the CaR is implicated in Sr\(^{2+}\)-induced NF-κB translocation, which decreased from 49 ± 2% to 20 ± 4% 30 min following addition of Sr\(^{2+}\) (25 mM) (p < 0.01, Fig. 5D). Preincubation of the cells for 30 min with a well characterized inhibitor of NF-κB (5 μM Ro106-9920) (30) abrogated the ability of extracellular Sr\(^{2+}\) (from 15 to 25 mM) to stimulate mature rabbit osteoclast apoptosis, confirming the role played by NF-κB activation in Sr\(^{2+}\)-induced osteoclast apoptosis (Fig. 5E). Because Sr\(^{2+}\) and Ca\(^{2+}\) seem to stimulate apoptosis in rabbit osteoclasts by different intracellular signaling pathways, i.e. PKCβII- and IP\(_3\)-depend- ent, respectively, and because they both stimulate different patterns of NF-κB translocation (19), we speculated that Ca\(^{2+}\) and Sr\(^{2+}\) may act additively, and therefore we introduced them together in culture. Interestingly, Sr\(^{2+}\) was able to potentiate the effects exerted by Ca\(^{2+}\) on NF-κB nuclear translocation, which rapidly reached and remained at a plateau of 50–55% as compared with the effects of Sr\(^{2+}\) or Ca\(^{2+}\) alone (Fig. 5F).

Similar results were obtained using the assay for apoptosis when various concentrations of Sr\(^{2+}\), from 12 to 24 mM, were introduced into the culture medium in combination with various concentrations of Ca\(^{2+}\), from 1.8 mM (the concentration of Ca\(^{2+}\) already present in the culture medium) to 20 mM (Fig. 6A). Taken together, these data strongly suggest that Sr\(^{2+}\) potentiates the effects of Ca\(^{2+}\) on both nuclear translocation of NF-κB and induction of mature osteoclast apoptosis. To confirm that Ca\(^{2+}\) and Sr\(^{2+}\) act through parallel and converging signaling pathways, both pharmacological inhibition and the use of siRNA were used. As shown in Fig. 6B, when mature osteoclasts incubated with both Ca\(^{2+}\) (20 mM) and Sr\(^{2+}\) (25 mM) were cultured with pharmacological inhibitors of Ca\(^{2+}\)-induced osteoclast apoptosis (2-APB (19)) and/or inhibitors of Sr\(^{2+}\)-induced osteoclast apoptosis (calphostin C or staurosporine), there was significant inhibition of Ca\(^{2+}\)- and Sr\(^{2+}\)-induced osteoclast apoptosis. Notably, calphostin C and staurosporine both potentiated the effect of 2-APB on Ca\(^{2+}\) and Sr\(^{2+}\)-induced apoptosis of mature osteoclasts (Fig. 6B). Osteoclast apoptosis was also assessed following transfection with siRNAs specific for the CaR or PKCβII or with scrambled siRNA. CaR-siRNA-transfected cells were shown to be resistant to Sr\(^{2+}\), Ca\(^{2+}\), and Sr\(^{2+}\)- induced apoptosis (Fig. 7B) as compared with osteoclasts trans- fected with the scrambled siRNA (Fig. 7A). Interestingly, in cells

**FIGURE 2.** Sr\(^{2+}\)-induced apoptosis of rabbit mature osteoclasts depends on activation of the CaR. A, increasing levels of Sr\(^{2+}\) (up to 25 mM) stimulate, to varying extents, apoptosis of β-galactosidase (β-Gal)- and DN-CaR-transfected mature rabbit osteoclasts. When treated with 20 and 25 mM Sr\(^{2+}\), apoptosis of DN-CaR-transfected osteoclasts is significantly reduced by about 36 and 30% compared with that observed in β-galactosidase-transfected cells. Data are expressed as means ± S.E. of three independent experiments (n = 18). *p < 0.05, and **p < 0.01, compared with DN-CaR-transfected osteoclasts within the group. $$$p < 0.001 compared with controls (β-galactosidase-transfected cells cultured for 48 h in the absence of Sr\(^{2+}\)). B, we assessed the role of PLC and IP\(_3\) signaling in Sr\(^{2+}\)-induced osteoclast apoptosis, using two well described pharmacological blockers (i.e. U73122 (10 μM) and SKF-96365 (10 μM)). Data are expressed as means ± S.E. of three independent experiments. ***p < 0.001 compared with osteoclasts cultured for 48 h with vehicle.

**FIGURE 3.** DAG analogue and phorbol esters stimulate apoptosis of mature osteoclasts. A, increasing concentrations of OAG, from 50 to 100 μM, stimulate in a dose-dependent manner the apoptosis of mature osteoclasts after 48 h of culture. Data are expressed as means ± S.E. of three independent experiments (6 wells/condition were counted for each experiment). ***, p < 0.001 compared with control. B, increasing concentrations of PMA, from 2.5 to 10 μM stimulate in a dose-dependent manner apoptosis of mature osteoclasts after 48 h of culture. Data are expressed as means ± S.E. of three independent experiments (6 wells/condition were counted for each experiment). ***, p < 0.001 compared with control. C, increasing concentrations of PDBeu, from 0.01 to 1 mM stimulate in a dose-dependent manner apoptosis of mature osteoclasts after 48 h of culture. Data are expressed as means ± S.E. of three independent experiments (6 wells/condition were counted for each experiment). **, p < 0.01, and ***p < 0.001, compared with control.
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FIGURE 4. **Sro**

transfected with PKCβII siRNA, only Ca\(^{2+}\) (20 mM) was able to stimulate osteoclast apoptosis when introduced alone into culture or in combination with Sr\(^{2+}\), consistent with our earlier observed difference in signaling between Sr\(^{2+}\) and Ca\(^{2+}\) (Fig. 7C).

DISCUSSION

In contrast to other therapies for osteoporosis, strontium ranelate exerts both anti-catabolic and anabolic effects on bone cells. Such an uncoupling action enables strontium ranelate to rebalance bone turnover in favor of bone formation in patients with osteoporosis (31–33). To elucidate the molecular mechanisms by which strontium ranelate inhibits bone resorption, we focused our attention on its effects on osteoclast apoptosis, one of the three important steps through which osteoclast bone resorption can be physiologically regulated (20). We demonstrated that Sr\(^{2+}\) significantly induces apoptosis of mature rabbit osteoclasts by a mechanism depending, at least in part, on stimulation of the CaR, activation of PLC, and nuclear translocation of NF-κB. However, unlike Ca\(^{2+}\), the action of Sr\(^{2+}\) on the apoptosis of mature rabbit osteoclasts appears to depend on the activation of PKCβII and not on IP\(_3\) signaling, showing that different intracellular signaling pathways mediate the effects of the two ions (Fig. 8).

To date we do not understand how Ca\(^{2+}\) and Sr\(^{2+}\) trigger different intracellular signaling pathways by stimulating a common receptor, the CaR. Specific extracellular binding sites for different agonists of the CaR have been identified (34). It could be hypothesized that distinct, biochemically defined binding site(s) for Sr\(^{2+}\) and Ca\(^{2+}\) exist within the extracellular domain of the CaR that would activate different signaling pathways upon binding of the respective ions to the receptor. Indeed, the CaR is known to activate multiple G proteins involved in distinct signaling pathways (35). The CaR couples to G\(_{q}\), which inhibits activation of adenylate cyclase, to G\(_{q}\), which stimulates PLC, to G\(_{q/11}\) which stimulates phospholipase D, and to the formation of G\(_{q/11}\) subunits, which stimulate phosphatidylinositol 3-kinase. The importance of CaR coupling to different G proteins in Sr\(^{2+}\) and Ca\(^{2+}\)-induced apoptosis requires further investigation.

The targeting of Sr\(^{2+}\) to bone mineral could confer upon this agent a specific and direct action on osteoclasts out of proportion to its circulating level. However, the actual concentration of Sr\(^{2+}\) at sites of bone resorption is not known. As a consequence of both osteoclastic bone resorption and either ionic exchange with Ca\(^{2+}\) or adsorption onto the hydroxyapatite crystal, Sr\(^{2+}\) may be taken up at the bone tissue level at concentrations substantially higher than those present in the circulation and other extracellular fluids. Consequently, in strontium ranelate-treated patients, the concentrations of Sr\(^{2+}\) and presumably Ca\(^{2+}\), could increase simultaneously in the subosteoclastic compartment during the bone resorptive process. Indeed, the level of Ca\(^{2+}\) in the immediate vicinity of osteoclasts has been measured to be as high as 8–40 mM, although similar studies measuring local levels of Sr\(^{2+}\) near resorbing osteoclasts have not been carried out using the bones of Sr\(^{2+}\)-treated animals. We recently demonstrated that increasing levels of Ca\(^{2+}\) up to 20 mM rapidly leads to apoptosis of mature osteoclasts through a classical caspase-3 and caspase-9-dependent mechanism. Ca\(^{2+}\) (20 mM)-induced osteoclast apoptosis at least partially involves the CaR, which triggers a PLC-dependent release of intracellular calcium stores via the IP\(_3\) signaling pathway (19). In the present study, we have demonstrated that Sr\(^{2+}\)-induced osteoclast apoptosis depends, at least in part, on stimulation of the CaR and on activation of PLC but not on IP\(_3\) signaling, suggesting that PKC activation could be involved instead.

In humans, at least 12 different PKC isoforms have been identified. These include: α, βI, βII, γ, δ, ε, ζ, η, τ, ι, λ, and μ. All
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FIGURE 5. Sr\(^{2+}\) stimulates nuclear translocation of NF-κB in mature rabbit osteoclasts. As illustrated in A and B (confocal imaging: ×200), osteoclasts were only rated positive for nuclear translocation of NF-κB when the NF-κB fluorescent labeling of one or more of the nuclei exceeded that of the cytoplasm (A). C, β-galactosidase (bGal)-transfected rabbit osteoclasts were treated with RANKL (50 ng/ml) or its vehicle for various times up to 12 h. Coverslips were then fixed at the indicated times, and p65 localization was determined by immunofluorescence. Data are expressed as means ± S.E. of the percentage of osteoclasts exhibiting nuclear localization of NF-κB on each coverslip and are representative of three to six independent experiments. **, p < 0.01, and ***, p < 0.001, compared with vehicle-treated, β-galactosidase-transfected osteoclasts. D, β-galactosidase- or DN-CaR-transfected rabbit osteoclasts were treated with 25 mM Sr\(^{2+}\) or its vehicle for up to 12 h. Data are representative of three to six independent experiments. **, p < 0.01, and ***, p < 0.001, compared with β-galactosidase-transfected osteoclasts cultured without Sr\(^{2+}\) (bGal + vehicle). S, p < 0.05 compared with β-galactosidase-transfected osteoclasts cultured with 25 mM Sr\(^{2+}\) (bGal + Sr 25 mM). E, we assessed the effect of a well-characterized inhibitor of NF-κB activation (Ro106-9920) on Sr\(^{2+}\)-induced osteoclast apoptosis. Data are expressed as means ± S.E. of three independent experiments (6 wells/condition were counted for each experiment). **, p < 0.01 compared with osteoclasts cultured for 48 h in the presence of Sr\(^{2+}\) at concentrations ranging from 15 to 25 mM. F, mature osteoclasts were treated with 24 mM Sr\(^{2+}\), 20 mM Ca\(^{2+}\), alone or with both together, for up to 12 h. Data are representative of three independent experiments. **, p < 0.01, and ***, p < 0.001, compared with Ca\(^{2+}\) (20 mM)-treated cells.

of them differ in their primary structure, tissue distribution, subcellular localization, mode of action in vitro, response to extracellular signals, and substrate specificity. PKCs α, β, II, and γ comprise the so-called “conventional” family, and their activities are Ca\(^{2+}\)- and phospholipid-dependent, whereas PKCs δ, ε, η, and τ comprise the “novel” family and are Ca\(^{2+}\)-independent but phospholipid-dependent. PKCs ζ, μ, and ι form the “atypical” family, which are activated neither by phorbol esters nor by DAG. Osteoclasts are known to express at least seven different PKC isoforms (α, β, II, δ, ε, ζ, and ι), which may serve as important inhibitory effectors during the process of bone resorption (36, 37, 38). Herein, we demonstrated that both a DAG analogue and phorbol esters trigger, in a dose-dependent manner, apoptosis of mature osteoclasts, suggesting for the first time that DAG-PKC signaling regulates the apoptotic process in mature osteoclasts. It is of note that experiments using phorbol esters as well as DAG analogues have already suggested that PKC pathways are important inhibitory signals in regulating the resorptive activities of rat and chicken osteoclasts (39). However, none of these studies focused on osteoclast apoptosis, which is a key step involved in terminating the bone resorption cycle. Notably, concentrations of active phorbol esters substantially higher than those generally used in experiments assessing the role of PKC (i.e., 10–100 nM PMA and μM levels of PDBo) were required to significantly increase the apoptosis of mature osteoclasts in our studies. The reasons underlying the apparently reduced sensitivity of the apoptotic process to PKC activators in mature osteoclasts need further investigation. One of these might be the relatively long duration of the experiments (~48 h) during which the phorbol ester might be degraded or absorbed nonspecifically and become unavailable to the PKC isoforms. Of note in this regard, how-
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FIGURE 6. \(\text{Sr}^{2+}\) potentiates \(\text{Ca}^{2+}\)-induced osteoclast apoptosis. A, purified osteoclasts were cultured for 48 h in the presence of increasing concentrations of \(\text{Sr}^{2+}\), from 0 to 24 mM and in the presence of various concentrations of \(\text{Ca}^{2+}\) (1.8, 8, 15, and 20 mM). Results are expressed as the mean ± S.D. of two independent experiments (6 wells/condition were counted for each experiment). B, mature rabbit osteoclasts were cultured for 48 h in the presence of both \(\text{Sr}^{2+}\) (25 mM) and \(\text{Ca}^{2+}\) (20 mM), with or without 2-APB, calphostin C (Calphoc), and staurosporine (Staurosp), which were added to the culture medium at 50 \(\mu\text{M}\), 1 mM, and 0.1 mM, respectively. Data are expressed as means ± S.E. of three independent experiments (6 wells/condition were counted for each experiment). ***, \(p < 0.001\) compared with untreated cells. $$$, \(p < 0.001\), compared with \(\text{Sr}^{2+}\)-treated osteoclasts. #, \(p < 0.05\); ##, \(p < 0.01\).

FIGURE 7. Respective roles of the CaR and PKCβIII in \(\text{Sr}^{2+}\)- or \(\text{Ca}^{2+}\)-induced osteoclast apoptosis. Mature rabbit osteoclasts transfected with scrambled siRNAs (A) or with siRNAs specific for the CaR (B) or PKCβIII (C) were cultured for 48 h in the presence of \(\text{Sr}^{2+}\) (25 mM), \(\text{Ca}^{2+}\) (20 mM), or both. Data are expressed as the means ± S.E. of three independent experiments (6 wells/condition were counted for each experiment). **, \(p < 0.01\); ***, \(p < 0.001\); ns, non significant.

ever, our results obtained utilizing siRNA and PKC inhibitors, which strongly support the involvement of PKC in the induction of apoptosis of mature osteoclasts.

Both nonspecific and specific antagonists of PKC inhibited \(\text{Sr}^{2+}\)-induced osteoclast apoptosis, suggesting that a common

targeted PKC(s), i.e. PKC\(\alpha\) and/or PKC\(\beta\), was or were involved in the effects of \(\text{Sr}^{2+}\). To confirm this hypothesis, we utilized specific siRNAs to down-regulate expression of the mRNAs for PKC\(\alpha\), PKC\(\beta\), and PKC\(\beta\). Only the siRNAs targeting PKC\(\beta\) were able to inhibit \(\text{Sr}^{2+}\)-induced apoptosis, indicating that the PKC\(\beta\) isoenzyme is involved in \(\text{Sr}^{2+}\)-induced osteoclast apoptosis, whereas PKC\(\alpha\) and PKC\(\beta\) are not. A full pharmacological assessment may be of help in confirming this result in future studies. It is of note that staurosporine and calphostin C were unable to reduce \(\text{Ca}^{2+}\)-induced osteoclast apoptosis, confirming that \(\text{Sr}^{2+}\) and \(\text{Ca}^{2+}\) stimulate mature osteoclast apoptosis by activating different intracellular signaling pathways.

As with \(\text{Ca}^{2+}\) (19), \(\text{Sr}^{2+}\)-induced NF-\(\kappa\)B activation in mature rabbit osteoclasts, a process that is downstream of the stimulation of the CaR. Similarly, \(\text{Sr}^{2+}\)-evoked activation of NF-\(\kappa\)B seems to be linked directly to apoptosis of mature osteoclasts. Thus, NF-\(\kappa\)B activation may be considered as a transcription factor important for survival of mature rabbit osteoclasts, as we and others have suggested previously (19, 40). Notably, the patterns of \(\text{Sr}^{2+}\)- and \(\text{Ca}^{2+}\)-evoked NF-\(\kappa\)B nuclear translocation differ from one another. That is, \(\text{Sr}^{2+}\) exerted a biphasic effect on NF-\(\kappa\)B activation, whereas \(\text{Ca}^{2+}\)-elicited nuclear translocation exhibited a single peak with maximal activation occurring at 1 h. It appears possible that this difference may be related to the different intracellular signaling pathways used by \(\text{Sr}^{2+}\) and \(\text{Ca}^{2+}\) to trigger mature osteoclast apoptosis. Further studies are needed to specifically address the role played by NF-\(\kappa\)B activation in the regulation of mature osteoclast apoptosis. Activation of other transcription factors, such as AP-1 and NFATc, also will require further study in order to clarify their relative importance in \(\text{Sr}^{2+}\) and \(\text{Ca}^{2+}\)-induced osteoclast apoptosis.

In combination, \(\text{Ca}^{2+}\) and \(\text{Sr}^{2+}\) increase the apoptosis of mature osteoclasts to a greater extent than does \(\text{Ca}^{2+}\) alone, suggesting that resorption of bone containing \(\text{Sr}^{2+}\) at sites of
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FIGURE 8. Schematic diagram summarizing the role played by the CaR in both Ca\(^{2+}\)- and Sr\(^{2+}\)-induced apoptosis of mature osteoclasts. Upon stimulation by extracellular calcium, the CaR activates PLC, which is responsible for the translocation of NF-κB from the cytoplasm to the nucleus in mature osteoclasts, in an IP\(_3\)-dependent manner. Upon stimulation by extracellular strontium, the CaR also activates PLC, which, in turn, is responsible for activation of the DAG-PKC\(\beta\)II signaling pathway, which in turn promotes translocation of NF-κB from the cytoplasm to the nucleus in mature osteoclasts in an IP\(_3\)-independent manner. Most likely in association with other transcription factors, Ca\(^{2+}\) and Sr\(^{2+}\)-induced activation of NF-κB can potentiate their respective effects, leading to enhanced apoptosis of mature osteoclasts.

Bone breakdown would produce apoptosis to a greater extent than Ca\(^{2+}\) alone. This effect could be explained by the different intracellular signaling cascades used by the two divalent ions to stimulate osteoclast apoptosis. Thus, our data indicate that both divalent cations act through the same receptor, the CaR. To date, we do not know whether Ca\(^{2+}\) and Sr\(^{2+}\) bind simultaneously to the same cellular pool of the CaR or activate different subcellular pools of the receptor, which may differ in the manner and/or extent to which they couple to different intracellular signaling pathways regulating apoptosis, for example. We know that the CaR belongs to family C of the superfamily of G protein-coupled receptor, which share a large extracellular domain that is thought to contain key Ca\(^{2+}\)-binding sites (41). Given the Hill coefficient of the CaR, three to five Ca\(^{2+}\) ions are likely to bind cooperatively to the CaR homodimer (42, 43). Whether or not the CaR binding sites for Ca\(^{2+}\) also bind Sr\(^{2+}\) or whether there are distinct binding sites for Ca\(^{2+}\) and Sr\(^{2+}\) within the extracellular binding domain of the receptor (34) requires further investigation. Progress in understanding the mechanisms mediating the activation of the CaR by Ca\(^{2+}\) and Sr\(^{2+}\), as well as by Mg\(^{2+}\), polyamines, and amino acids, may significantly elucidate how the CaR functions.

In conclusion, we have demonstrated in this study that the CaR is involved in Sr\(^{2+}\)-induced osteoclast apoptosis through a signaling pathway that depends on PLC, PKC\(\beta\)II, and NF-κB activation, which may represent a new pathway whereby osteoclast apoptosis can be regulated. Our results suggest that Sr\(^{2+}\), acting on the CaR on osteoclasts through a mechanism similar to but differing in certain respects from that of Ca\(^{2+}\), could potentiate the effects of Ca\(^{2+}\) and lead to more marked down-regulation of osteoclastic bone resorption through osteoclast apoptosis. This effect may be directly implicated in the anti-resorptive action of strontium ranelate on bone in vivo.

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