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Strontium ranelate stimulates the activity of bone-specific alkaline phosphatase: interaction with Zn^{2+} and Mg^{2+}

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Abstract Strontium ranelate (SR) is an orally administered and bone-targeting anti-osteoporotic agent that increases osteoblast-mediated bone formation while decreasing osteoclastic bone resorption, and thus reduces the risk of vertebral and femoral bone fractures in postmenopausal women with osteoporosis. Osteoblastic alkaline phosphatase (ALP) is a key enzyme involved in the process of bone formation and osteoid mineralization. In this study we investigated the direct effect of strontium (SR and $SrCl_2$) on the activity of ALP obtained from UMR106 osteosarcoma cells, as well as its possible interactions with the divalent cations Zn^{2+} and Mg^{2+}. In the presence of Mg^{2+}, both SR and $SrCl_2$ (0.05–0.5 mM) significantly increased ALP activity (15–66 % above basal), and this was dose-dependent in the case of SR. The stimulatory effect of strontium disappeared in the absence of Mg^{2+}. The cofactor Zn^{2+} also increased ALP activity (an effect that reached a plateau at 2 mM), and co-incubation of 2 mM Zn^{2+} with 0.05–0.5 mM SR showed an additive effect on ALP activity stimulation. SR induced a dose-dependent decrease in the Km of ALP (and thus an increase in affinity for its substrate) with a maximal effect at 0.1 mM. Co-incubation with 2 mM Zn^{2+} further decreased Km in all cases. These direct effects of SR on osteoblastic ALP activity could be indicating an alternative mechanism by which this compound may regulate bone matrix mineralization.

Keywords Bone-specific alkaline phosphatase · Magnesium · Zinc · Strontium ranelate · Osteoblasts

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Introduction

Strontium ranelate (SR) is an orally administered anti-osteoporotic agent that includes two strontium ions and an organic moiety (ranelic acid) Saidak and Marie (2012) Sr^{2+} can partially substitute Ca^{2+} in hydroxyapatite, and thus be incorporated into bone. SR has been shown to effectively reduce the risk of vertebral and femoral bone fractures in postmenopausal women with osteoporosis (Meunier et al. 2004; Reginster et al. 2005). In vitro, strontium exerts a dual effect on bone cells: it increases osteoblast-mediated bone formation, while decreasing osteoclastic bone resorption (Saidak and Marie 2012; Takaoka et al. 2010).These combined actions correlate with its beneficial effects on bone mass, bone quality and bone resistance in animal models of metabolic bone disease, as well as in osteoporotic patients. In osteoblasts, strontium stimulates cellular proliferation and differentiation via the

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calcium sensing receptor and the Wnt/beta-catenin pathway (Takaoka et al. 2010).

Mature osteoblasts express high levels of tissue specific alkaline phosphatase (ALP), a key enzyme involved in the process of bone formation and osteoid mineralization (Whyte 1994). This marker of osteoblastic differentiation is a metallo-enzyme anchored to both the plasma membrane and to secreted matrix vesicles (Van Hoof and De Broe 1994). ALP is a phosphomonoesterase, whose catalytic activity depends on a multimeric configuration. It requires Mg^{2+} as a cofactor and includes two Zn^{2+} ions per monomer to stabilize its tertiary structure. Because ALP is an exoenzyme that faces the extracellular compartment, it is conceivable to hypothesize that its activity and function could be modulated by environmental conditions and pharmacological treatments. For instance, we have previously provided evidence for the alteration of ALP activity and structure as a consequence of its in vitro glycation (McCarthy et al. 1998) and of its co-incubation with N-containing bisphosphonates (Vaisman et al. 2005).

Sr^{2+}, when orally administered as SR, is partially incorporated into bone (Dahl et al. 2001). After a treatment of 4 weeks or more, plasma levels and bone content of strontium reach a plateau, with a very good correlation between them both. However, strontium content of bone can additionally vary according to its turnover and anatomical localization: for example, higher amounts of strontium are found in cancellous bone than in cortical bone.

In recent in vitro studies with MC3T3E1 osteoblastic cells, we have shown that Sr^{2+} can increase type 1 collagen production, ALP specific activity and expression of intracellular markers such as extracellular-regulated kinases (ERK) and β-catenin, while decreasing secretion of the pro-inflammatory cytokines TNFα and IL1 (Fernandez et al. 2013). Sr^{2+} incorporated into the mineral lattice could directly interact with bone-ALP expressed by osteoblasts and/or present in secreted matrix vesicles, thus potentially influencing the process of osteoid mineralization. In the present study, we have investigated the direct effect of strontium (as ranelate and chloride) on the activity of osteoblast-derived ALP, as well as possible mechanisms that involve its interaction with the divalent cations Zn^{2+} and Mg^{2+}.

Materials and methods

Materials

SR was provided by Servier, Argentina. Dulbecco's modified Eagle's medium (DMEM), trypsin–EDTA, and fetal bovine serum (FBS) were from Gibco, (Life Technology, Buenos Aires, Argentina) and tissue culture disposable material was from Nunc (Buenos Aires, Argentina). Para-nitrophenyl phosphate (p-NPP), strontium chloride, wheat germ agglutinin (WGA) and other reagents of analytical grade were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

UMR106 rat osteosarcoma-derived cells were obtained from American Type Culture Collection (ATCC) (Rockville, MI, USA). Cells were grown in 75-cm^2 flasks at 37 °C in a humidified 5 % CO_2 atmosphere in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10 % FBS. When 70–80 % confluence was reached, cells were sub-cultured using 0.1 % trypsin–1 mM EDTA in Ca^{+2}–Mg^{+2}-free phosphate-buffered saline (PBS). After cells reached confluence, the monolayer was washed with PBS and solubilised in 0.1 % Triton X-100. After that, osteoblastic ALP was purified by its affinity for WGA. Briefly, a volume of total cell extract was incubated for 30 min with the same volume of WGA (5 mg/ml) (Broyles et al. 1998; Farley et al. 1994; Puche et al. 1988), after which the precipitate was separated by centrifugation and resuspended in an equal volume of PBS. Aliquots of ALP thus purified were used for evaluation of enzymatic activity as described below. We have previously characterized ALP from UMR106 cell extracts (Vaisman et al. 2005).

Alkaline phosphatase activity assay

The initial rate of ALP enzymatic activity from WGA-isolates was determined by spectrophotometric evaluation of hydrolysis of p-NPP to p-nitrophenol (p-NP) in a glycine–Mg buffer, pH 10.5, at 37 °C for 5 min. Alternatively, a glycine buffer without Mg was used. Formation of the product was assessed by absorbance

at 405 nm with a PG Instruments T60 UV–visible spectrophotometer. Under these conditions, the reaction proceeds linearly for 30 min. The effects of different doses of SR, $SrCl_2$ and/or $ZnCl_2$ were tested after their pre-incubation for 10 min at 37 °C with identical aliquots of the same osteoblastic extract. Results are expressed as the initial rate of bone-specific ALP activity (nmol pNP/min).

To determine if SR affects the affinity (Km) for p-NPP, we evaluated the production of p-NP after 2 and 5 min of reaction with an aliquot of the extract and increasing concentrations of SR, with or without Zn^{+2}, in a glycine—Mg buffer. The Km was calculated according to Lineweaver–Burk (Eq. 1)

$$\frac{1}{V} = \frac{K_m}{V_{\max}[S]} + \frac{1}{V_{\max}} \quad (1)$$

Where V is the reaction velocity, K_m is the Michaelis–Menten constant, V_{max} is the maximum reaction velocity and [S] is concentration of substrate.

Statistical analysis

For each experimental condition, at least three separate experiments were performed by triplicate. Data are expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t* test, and a $p < 0.05$ was considered to be significant.

Results

We have previously characterized ALP from UMR106 cell extracts by heat inactivation, showing that this bone isoform represents about 90 % of total cellular ALP (Vaisman et al. 2005). Among other characteristics, ALP iso-enzymes differ in their post-translational glycosylation and thus in their affinity for specific lectins. In particular, bone-ALP shows high affinity for WGA, and thus can be easily separated from other iso-enzymes by selective precipitation with this lectin (Farley et al. 1994). In the present study we investigated the possible modulation of the activity of WGA-precipitated ALP by SR and $SrCl_2$. Under our experimental conditions, SR significantly stimulated the initial rate of p-NPP hydrolysis in a dose-dependent manner (Fig 1a; Table 1) as from 0.05 mM of SR. The maximal effect at the evaluated doses was observed for 0.5 mM of SR (66 % over basal). ALP activity was also increased about 30 % over basal by 0.01–0.1 mM $SrCl_2$, although in this concentration range the effect of $SrCl_2$ did not appear to be dose-dependent (Fig 1b; Table 1).

Since bone-specific ALP requires Mg^{2+} as a cofactor, we investigated whether Sr^{2+} could be replacing this ion to stimulate ALP activity. To address this question, we examined the possible effect of different doses of SR on bone-specific ALP activity in the absence of Mg^{2+} in the incubation buffer. As can be seen in Fig 1c and Table 1, Mg^{2+}-deprivation significantly inhibited ALP activity by about 30 %, and this inhibition could not be overcome by co-incubation with 0.01–0.5 mM SR. In fact, 0.5 mM SR actually tended to inhibit (by about 50 %) the remaining bone-specific ALP activity, although this did not reach statistical significance.

We next examined the effect on bone-specific ALP activity of increasing cofactor (Zn^{2+}) concentration. As can be seen in Fig 2a, b, Zn^{2+} increased ALP activity in a dose-dependent manner, with a maximal stimulation reached at 2 mM $ZnCl_2$ (220 % of basal, $p < 0.001$).

Results presented in Figs 1 and 2 show that in our experimental conditions both Sr^{2+} and Zn^{2+} stimulate bone-specific ALP activity. Hence, we sought to determine if there might be synergistic or additive interactions between both ions. In further experiments we evaluated the effect on ALP activity of 2 mM Zn^{2+} plus two doses (0.05 or 0.5 mM) of SR. The results are shown in Fig 3. In the presence of Zn^{2+}, 0.05 mM SR stimulated ALP activity (267 % of basal, $p < 0.001$), and this effect was significantly greater than the effects of 2 mM Zn^{2+} alone ($p < 0.05$) or of 0.05 mM SR alone ($p < 0.001$). Likewise in the presence of Zn^{2+}, 0.5 mM SR significantly increased bone-ALP activity over control (306 % versus basal, $p < 0.001$) as well as over 2 mM Zn^{2+} alone ($p < 0.001$). Our results suggest an additive effect for both divalent cations (Sr^{2+} and Zn^{2+}) on bone-ALP activity.

In order to characterize the effect of SR on the kinetic parameters of ALP, the WGA-isolates were incubated with different concentrations of SR (0, 0.05, 0.1 and 0.5 mM) in the absence or presence of 2 mM Zn^{2+}. The initial rates were determined with increasing concentrations of pNPP and the values were plotted as Michaelis–Menten curves in Fig. 4. Our results show that SR on its own dose-dependently increased ALP reaction velocity. In addition, co-

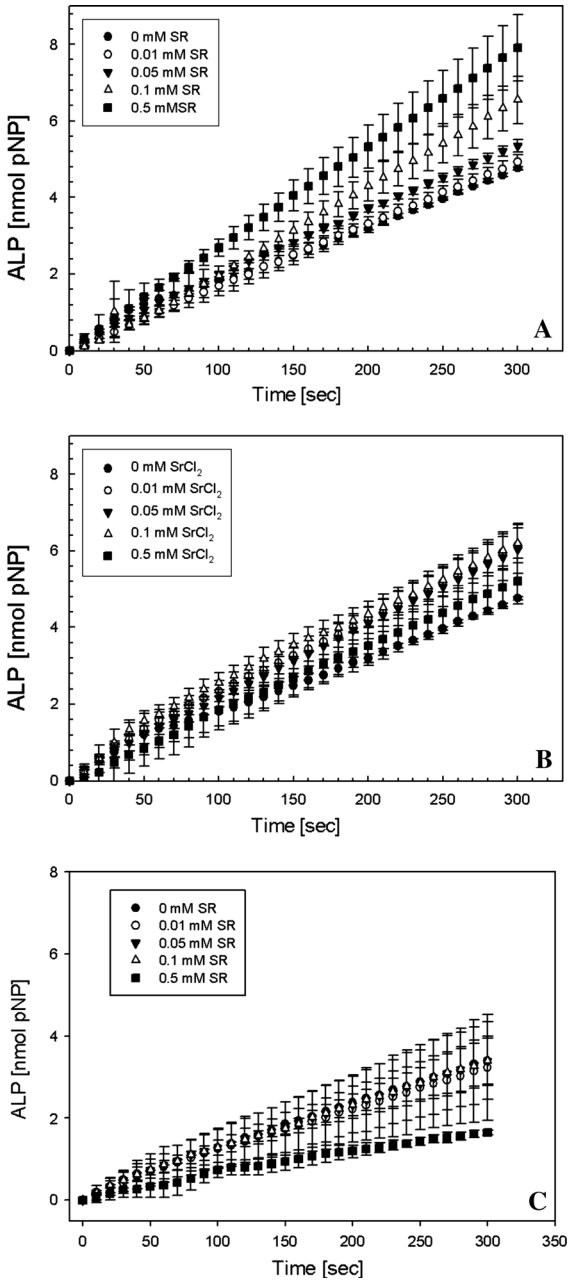

Fig. 1 Direct effect of strontium on alkaline phosphatase activity. **a** SR stimulates ALP activity in a dose-dependent manner, while **b** $SrCl_2$ stimulates ALP activity similarly at all doses tested. **c** Mg^{2+}-deprivation inhibited ALP activity and this inhibition could not be overcome by co-incubation with 0.01–0.5 mM SR

Table 1 Initial rate of ALP activity (nmol pNP/min)

Sr^{2+} (mM)	SR	$SrCl_2$	SR w/o Mg
0	0.95 ± 0.01	0.95 ± 0.01	0.68 ± 0.09*
0.01	0.98 ± 0.033	1.23 ± 0.08*	0.66 ± 0.12*
0.05	1.07 ± 0.03*	1.21 ± 0.13	0.65 ± 0.18*
0.1	1.31 ± 0.10*	1.24 ± 0.08*	0.68 ± 0.08*
0.5	1.58 ± 0.14**	1.04 ± 0.12	0.38 ± 0.13**

Difference versus Basal (without Sr^{2+}, with Mg^{2+}): * $p < 0.05$, ** $p < 0.02$

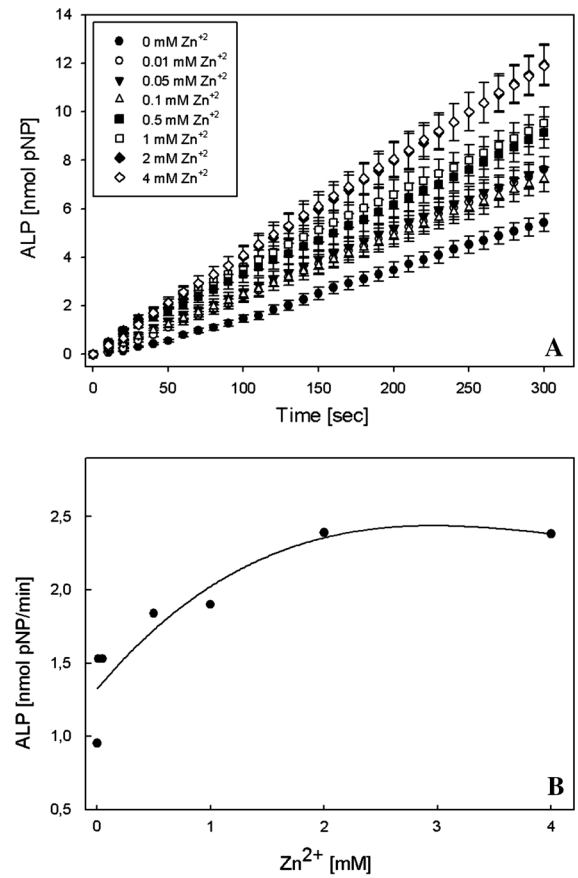

Fig. 2 Zinc is a cofactor for ALP activity. Zn^{+2} increases ALP activity in a time (**a**) and dose (**b**) dependent manner

incubation with Zn^{2+} potentiated the effect of SR on ALP initial rate. Using the equation of Lineweaver–Burk we determined Km for each experimental condition (Fig. 5). As can be seen, SR induced a dose-dependent decrease in Km (and thus an increase in the affinity of ALP for its substrate) with a maximal effect at 0.1 mM. Co-incubation with 2 mM Zn^{2+} further decreased Km in all cases.

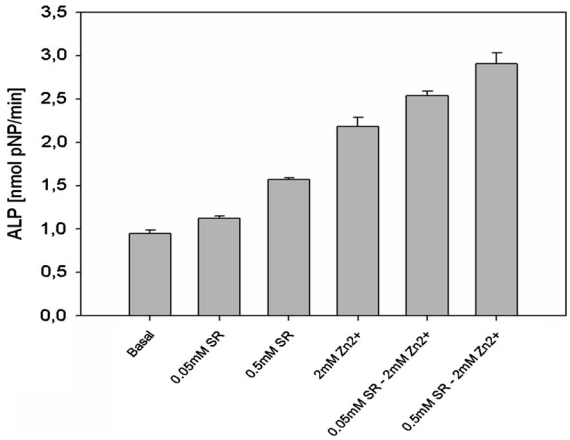

Fig. 3 Strontium ranelate and zinc have an additive effect on ALP activity. Different doses of SR and 2 mM Zn^{+2} stimulate ALP activity. The combination of both drugs results in an additive increase in ALP activity

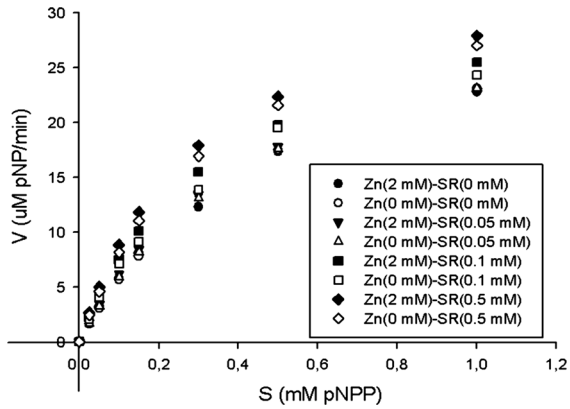

Fig. 4 Effects of Zn^{2+} and SR on kinetics of ALP-catalyzed hydrolysis of pNPP. The initial rates of hydrolysis of increasing concentrations of pNPP in the presence of 0 mM Zn^{2+} (*white symbols*) or 2 mM Zn^{2+} (*black symbols*) and different concentrations of SR were determined

Discussion

Recent reports have demonstrated that the beneficial effects of SR on bone metabolism result from differential effects of this agent on the recruitment and activity of both osteoclasts and osteoblasts. SR increases the replication of pre-osteoblastic cells as well as the activity of functional osteoblasts, resulting in increased bone matrix synthesis (Saidak and Marie 2012). On the other hand, SR inhibits osteoclast formation by antagonizing RANKL-induced nuclear

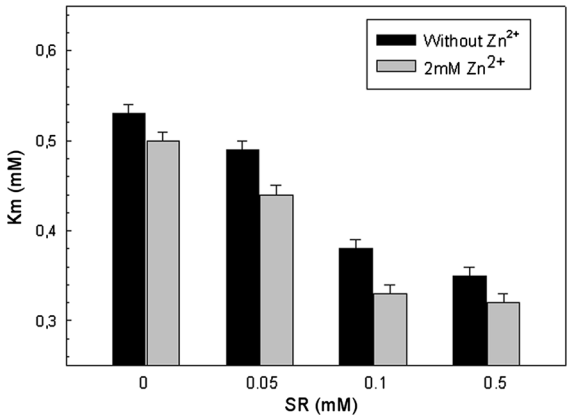

Fig. 5 Representation of the Michaelis–Menten constant (Km) of ALP for different concentrations of SR and/or Zn^{2+}

translocation of NF-κB and AP-1 transcription factors (Caudrillier et al. 2010). Although many effects of SR are probably mediated by interaction of Sr^{2+} with the calcium-sensing receptor and activation of pathways such as ERK and Wnt/β-catenin (Takaoka et al. 2010), recently Yamaguchi and Weitzmann (2012) have suggested that certain biological actions of SR could be related to low doses of the intact (undissociated) molecule. However, no studies have reported the direct effect of Sr^{2+} (and specifically SR) on osteoblast-derived phosphatases. In the present study we sought to investigate the possible direct effect of SR on bone-specific ALP activity, as an alternative mechanism by which this compound could regulate matrix mineralization.

We have shown that Sr^{2+} exerts a direct stimulation of osteoblast-derived and WGA-precipitated ALP activity. At the concentrations evaluated, this effect was dose-dependent when the agent tested was SR but not with $SrCl_2$. The differences observed between both compounds at low concentrations could be due to differing levels of free Sr^{2+} as a consequence of a lower dissociation of SR. In fact, ALP activity with 0.01 mM SR (Table 1) was similar to that with 0.001 mM $SrCl_2$ (data not shown), which is in agreement with an incomplete dissociation of SR. The concentrations of strontium tested in this study were chosen based on our (and other) previous studies on its in vitro effects on osteoblasts in culture (Yamaguchi and Weitzmann 2012; Fernandez et al. 2010; Fernandez et al. 2013; Barbara et al. 2004). In addition, the strontium serum concentration in post-

menopausal women with osteoporosis in long-term treatment with SR has been reported to be in the range of 0.1–0.2 mM (Marie 2008) Our previous results (Fernandez et al. 2013) and those of other authors show that levels of SR around 0.1 mM are optimal for increasing osteoblast activity and function. This coincides with our present results showing that 0.1 mM of SR lowers the Km of ALP, thus increasing affinity of the enzyme for its experimental substrate (Fig. 5a, b). Under normal calcium dietary intake, strontium incorporation into the bone matrix correlates with serum strontium levels. Using a synchrotron radiation-induced micro X-ray fluorescence (SR m-XRF) method, concentrations of strontium were determined in bone tissue (Roschger et al. 2010; Fuchs et al. 2008). However, the strontium levels reported by this technique were expressed in relative values (wt %), and thus it is not possible to compare with the in vitro or in vivo serum levels of strontium, as has recently been emphasised by Roschger and coworkers (2011). Nevertheless, it is possible that strontium accumulated in the bone matrix could directly regulate osteoblast-derived ALP during the mineralization process, as our present observations suggest.

It is known that tissue non-specific ALPs are strongly regulated by Mg^{2+} and Zn^{2+} (Bosron et al. 1977). Our present results suggest that Mg^{2+} cannot be replaced by SR. Indeed, in our experimental conditions strontium-induced ALP activation required the presence of Mg^{2+} in the incubation media. In agreement with our results, previous investigations have proposed that Mg^{2+} is necessary for ALP activity because it maintains the tertiary structure of the enzyme (Anderson et al. 1975). On the other hand; we found an additive effect of SR and Zn^{2+} on ALP stimulation. In concordance with our observations, other authors (Llinas et al. 2006) have proposed the presence of a four-metal binding site in ALP. Based on crystallographic and sequence homology studies between the non-specific (present in bone) and placental ALP isoforms they proposed that this binding site should be occupied by calcium, which can be substituted by strontium. Other authors have also suggested that strontium could act as a competitor for binding site(s) in proteins such as ALP that are specific to cellular function (Fielding Fielding et al. 2012). Altogether, our present and previous observations suggest that strontium induces activation of ALP through its affinity for a specific metal-binding site, thus potentially regulating its activity during osteoid mineralization.

In conclusion, this study has demonstrated for the first time a direct stimulation of bone-derived ALP activity by strontium (and particularly by SR) that is partly due to a SR-induced increase in the affinity of this enzyme for its substrate.

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