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1,25-Dihydroxyvitamin D_3 Decreases Alkaline Phosphatase Activity in Cultures of Embryonic Chick Tibiae (42029)

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Abstract. The influence of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] on the alkaline phosphatase (AlPase) activity in cultures of chick embryo tibiae was determined. A dose-related, decreased release (30–47%) of AlPase from the bones was seen with the metabolite at 0.05–0.5 ng/ml of medium with a similar effect on the bone content of enzyme. The highest dose (1 ng/ml) decreased the bone content by 38% without further effect on AlPase release. Combining a low level of 1,25$(OH)_2D_3$ (0.05 ng/ml) with parathyroid hormone (PTH, 1 U/ml) reduced release of enzyme additively, but caused no greater decrease in bone content of activity than PTH alone. No effects of 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$, 0.5 ng/ml] on release or bone content of AlPase were found when this metabolite was added alone or in combination with PTH; however, 24,25$(OH)_2D_3$ did prevent the inhibition of release of AlPase when added with 1,25$(OH)_2D_3$. After a 1-day exposure to 1,25$(OH)_2D_3$, continued incubation in metabolite-free medium resulted in an 89% increase in bone content of AlPase. The results suggest that 1,25$(OH)_2D_3$, as well as PTH, may have regulatory roles in bone growth through their effects on AlPase.

Alkaline phosphatase (AlPase) activity is high in bone and has been associated with mineralization (1, 2) and with cells involved in the formation of skeletal components (3). We and others have demonstrated that parathyroid hormone (PTH) decreases AlPase activity in bones in organ culture (2, 4–6). Decreased AlPase activity has also been shown in isolated osteoblast-like cells treated with PTH (7, 8) and with 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (7). However, Manolagas *et al.* (9) have reported that 1,25$(OH)_2D_3$ stimulates AlPase activity in rat osteosarcoma cells and that neither 25-hydroxyvitamin D_3 nor 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] affect AlPase activity in these cells. Majeska and Rodan (10) have suggested that the apparent biphasic effect of 1,25$(OH)_2D_3$ is related to the degree of maturation of the cells. They speculate that the metabolite may increase AlPase activity in cultures of immature osteoblast-like cells by promoting differentiation of preosteoblasts to osteoblasts and may decrease activity in mature cultures by stimulating osteoblasts to mature into osteocytes with lower AlPase activity.

The purpose of the present study was to determine the effects of 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ on the AlPase activity of embryonic chick long bones *in vitro*. Possible interaction of these metabolites with PTH was tested as was the ability of the 1,25$(OH)_2D_3$-treated bones to recover after withdrawal of the metabolite.

Materials and Methods. *Organ culture.* Tibiae from 10-day White Rock chick embryos were harvested and placed in 25-ml Erlenmeyer flasks (1 bone/flask) containing 4 ml of culture medium. The flasks were gassed with 5% CO_2 in air, stoppered, and incubated for up to 4 days on a rocker platform in a 38°C incubator. All culture media were prepared by modification of Eagle's minimum essential medium as described by Ramp and Neuman (11) and contained bovine serum albumin (1 mg/ml). Medium Ca and P concentrations were both raised to 2 m*M* by addition of appropriate amounts of 100 m*M* $CaCl_2$ and 100 m*M* NaH_2PO_4. Bones from each embryo were paired such that one bone was incubated in medium

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containing test materials and the other was cultured in appropriate control medium. After culture, the bones were briefly rinsed in 0.15 *M* NaCl. Bones and medium were stored at −20°C until analyzed.

The vitamin D metabolites were kindly provided by Dr. Milan Uskokovic, Hoffmann-LaRoche Inc. Purified bovine PTH (550–800 U/mg) was obtained from Inolex. The vitamin D metabolites were dissolved in 95% ethanol and the vehicle for the PTH was 0.001 *N* HCl–0.15 *M* NaCl. Appropriate amounts of vehicles were added to control medium.

Analytical methods. Bones were homogenized in distilled water for 10 sec using a Polytron homogenizer (Brinkmann). Culture media and homogenates were assayed for AlPase activity at pH 9.8 using *p*-nitrophenylphosphate as substrate (12). The enzyme activity (A) is expressed as units per bone. One unit of activity represents one micromole of substrate hydrolyzed per hour at 38°C.

The significance levels of differences between paired control and treated bones or between unpaired treated bones were determined using Student's *t* test for paired or for unpaired data (one-tailed). A value of $P < 0.05$ was considered significant.

Results. *Dose-related effect of 1,25(OH)$_2$D$_3$ on AlPase release.* Tibiae cultured for 4 days with 1,25(OH)$_2$D$_3$ in the medium showed a dose-related decrease in the rate of release of AlPase activity (Fig. 1). The release was decreased 30, 39, and 47% with 1,25(OH)$_2$D$_3$ at 0.05, 0.1, and 0.5 ng/ml, respectively. No further decrease in release of AlPase activity was seen when bones were cultured with the metabolite at 1 ng/ml suggesting that the effect seen with 0.5 ng/ml was maximal.

Effects of 1,25(OH)$_2$D$_3$; 24,25(OH)$_2$D$_3$; and PTH alone and in combination. Comparing the activity of noncultured bones (2.28 ± 0.08 A/bone, mean ± SE, $N = 6$) with control bones cultured 4 days (11.4 ± 0.4 A/bone) shows that AlPase activity in the bones increased five-fold during the culture period (Fig. 2A). As reported earlier (4), these data suggest, but do not prove, that the tibiae actively synthesized AlPase *in vitro*. The addition of 1,25(OH)$_2$D$_3$ (0.05 ng/ml) to the medium of tibiae cultured for 4 days decreased both the release (38%) and the increase in bone content (26%) of AlPase (Figs. 2A, B). Regarding release, these results confirm those of the previous experiment. As previously reported (2, 4), PTH (1 U/ml) decreased both the release and bone content of AlPase. When both 1,25(OH)$_2$D$_3$ and PTH were in the medium, their effects on reduced AlPase release were additive and were significantly greater in combination than when added individually ($P < 0.05$). Combined 1,25-(OH)$_2$D$_3$ and PTH treatment did not demonstrate an additive reduction of bone AlPase content, since the value obtained was not significantly less than with PTH alone. When added alone, 24,25(OH)$_2$D$_3$ (0.5 ng/ml) did not affect AlPase release or content, but did significantly elevate the release of enzyme by 1,25(OH)$_2$D$_3$-treated bones as well as decrease their content ($P < 0.05$). Combination of 24,25(OH)$_2$D$_3$ with PTH or with 1,25-(OH)$_2$D$_3$ plus PTH did not influence AlPase release or bone content.

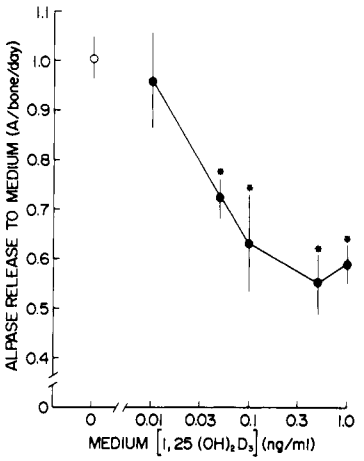

FIG. 1. Dose-related effect of 1,25(OH)$_2$D$_3$ on the release of AlPase from bone. Tibiae from 10-day chick embryos were paired between control medium (open circle) and 1,25(OH)$_2$D$_3$-containing medium (closed circles). The media were assayed for AlPase activity after culturing for 4 days. Since there was no significant difference in the release of enzyme from the groups of bones used as controls for each dose, these values were pooled and are represented by a single open circle ($N = 35$). Each closed circle represents the mean value of seven tibiae. Vertical lines represent the SE. $^*P < 0.01$ compared to paired control bones.

Recovery from 1,25(OH)$_2$D$_3$. Effects of the highest dose of 1,25(OH)$_2$D$_3$ tested (1 ng/ml

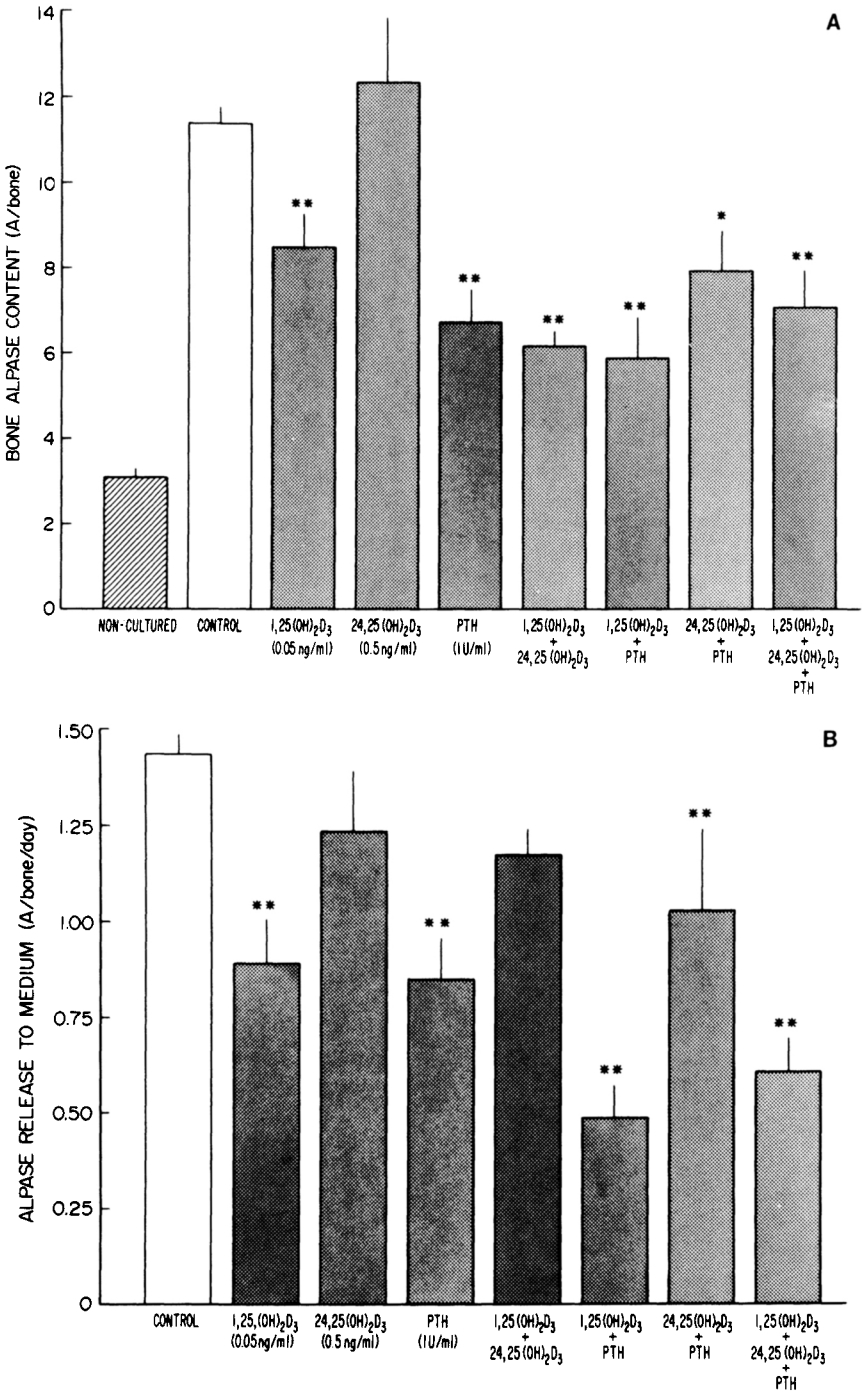

FIG. 2. Effects of vitamin D metabolites and PTH on the bone content (A) and release of AlPase (B) after culturing for 4 days. Tibiae from 10-day chick embryos were paired between control medium (open bars) and medium containing 1,25(OH)$_2$D$_3$; 24,25(OH)$_2$D$_3$; or PTH; either alone or in the combinations indicated (stippled bars). Each control bar represents the mean of 42 bones and each treated bar represents the mean of 6 bones (see Fig. 1). Vertical lines represent the SE. $^*P < 0.05$, $^{**}P < 0.01$ compared to paired control bones.

of medium) are shown in Fig. 3. Compared to noncultured bones, the activity of AlPase in the control tibiae more than doubled during the first day of culture and was increased more than 500% by the end of 4 days, again suggesting that AlPase is actively synthesized in this system. No effect of 1,25(OH)$_2$D$_3$ on bone content of enzyme was found after 1 day; however, tibiae treated with the metabolite showed a 38% decrease in AlPase activity compared to control bones cultured 4 days. When medium containing 1,25(OH)$_2$D$_3$ was removed after 1 day and replaced with metabolite-free medium for 3 days, the bones increased their AlPase activity to a level 89% greater than that in control tibiae. In an earlier study a similar effect was found when PTH was removed from cultures of tibiae from 7-day chick embryos (2). Although the AlPase content was markedly increased by removal of 1,25(OH)$_2$D$_3$, the release of the enzyme to the medium by these bones was reduced 21% during the 3-day recovery period (data not shown). This reduction closely approximated that seen in bones receiving 1,25(OH)$_2$D$_3$ during this period (26%). No consistent effect of 1,25-(OH)$_2$D$_3$ on release of enzyme at 1 day was found in this experiment.

Discussion. This study shows for the first time that 1,25(OH)$_2$D$_3$ can influence AlPase in whole bones by a direct action. Because of the numerous reports concerning this metabolite and its effects on isolated bone cells (7, 9, 10), our findings with whole bones may not be surprising. Although both increased (9) and decreased AlPase activity (7) have been reported for isolated cells in response to 1,25(OH)$_2$D$_3$, our findings demonstrate that the overall effect of the metabolite on rapidly growing long bones from chick embryos is to decrease AlPase activity; both in terms of bone content and release of the enzyme. Since AlPase has been associated with the growth and mineralization of bone (1–3), this effect of 1,25(OH)$_2$D$_3$ may represent an inhibitory function of the metabolite on bone mineral accretion. Such a response

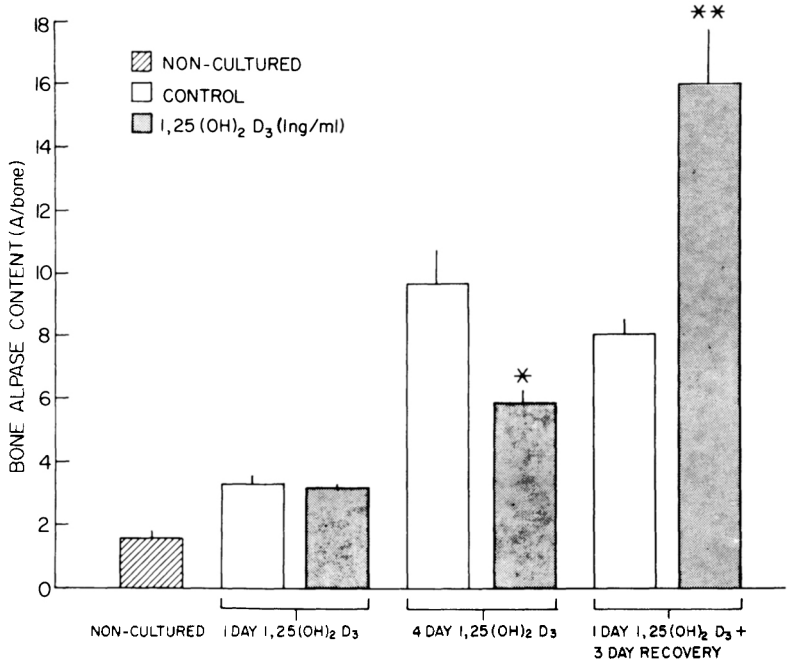

FIG. 3. Effects of exposure to 1,25(OH)$_2$D$_3$ followed by incubation without the metabolite on bone content of AlPase. Tibiae were assayed for AlPase content after treatment with 1,25(OH)$_2$D$_3$ for 1, 4, or 1 day followed by a 3-day recovery period. Corresponding control values for bones incubated without metabolite and for the AlPase content of noncultured tibiae are also shown. Vertical lines represent the SE. *$P < 0.01$, **$P < 0.001$ compared to control bones.

could compliment the well-known potent stimulatory effect $1,25(OH)_2D_3$ has on bone resorption (13, 14).

There is some evidence that $24,25(OH)_2D_3$ has a role in bone formation and mineralization (15, 16). If so, it might be expected that $24,25(OH)_2D_3$ would increase the level of AlPase activity in bone. However, when added alone no response to this metabolite was detected either in terms of enzyme release or bone content in our system, although other doses and exposure times might have yielded different results. In agreement with our findings, Wong *et al.* (7) reported no effect of $24,25(OH)_2D_3$ on the same bone cells that showed a marked decrease in AlPase activity in response to $1,25(OH)_2D_3$. Although having no effect when added alone, $24,25(OH)_2D_3$ did reduce the inhibition of AlPase release by $1,25(OH)_2D_3$-treated bone (Fig. 2B). It is unlikely that this effect could represent a mechanism for $24,25(OH)_2D_3$ to oppose inhibition of bone growth or stimulation of bone resorption by $1,25(OH)_2D_3$, since combining the metabolites further reduced the AlPase content in $1,25(OH)_2D_3$-treated bones (Fig. 2A).

The activity of bone AlPase in serum is often interpreted to reflect the bone content of AlPase (17). In the present study (Figs. 2A, B) and in earlier studies (2, 4), a similar relationship was demonstrated between cultured bones and medium. The physiological importance of the release of AlPase from bone is not known. The enzyme in the medium may be normally secreted from bone cells or may be due to release resulting from cell damage, such as might occur in an *in vitro* system. However, evidence obtained with the culture system used in the present investigation does not suggest the latter (18). When bone cells were killed with iodoacetate, the pattern of AlPase release was different than the decreased rate of release caused by PTH (18) or $1,25(OH)_2D_3$ (Fig. 3). Unlike PTH, the inhibitor caused a rapid loss of AlPase during the first 12 hr, after which no further release occurred, whereas the effect of PTH was to cause a gradual reduction in release over 48 hr. When ALPase release had ceased, whether as a result of PTH or iodoacetate, a fixed amount of activity (*ca.* 30%) remained in the bones suggesting that two pools of AlPase may exist; one which is releasable and one which is not.

Several explanations can be offered for the increased AlPase activity in bones exposed to $1,25(OH)_2D_3$ for 1 day followed by 3 days with the metabolite removed (Fig. 3): (1) since release of enzyme remained reduced during recovery, retention of AlPase by the bones increased during this period; (2) rebound from the inhibitory effect of a high dose of $1,25(OH)_2D_3$ (1 ng/ml of medium) stimulated AlPase synthesis in existing bone cells; (3) residual $1,25(OH)_2D_3$, possibly leached from the bones after the medium was changed to metabolite-free medium, resulted in a very low $1,25(OH)_2D_3$ level in the medium during the 3-day recovery and this concentration had a stimulatory effect either on AlPase synthesis or on the proliferation of osteoblasts containing elevated AlPase activity. Regarding the latter, low concentrations of $1,25(OH)_2D_3$ might stimulate bone formation in a manner similar to that seen after chronic exposure to low levels of PTH (19). For whatever reason, AlPase activity was clearly higher in tibiae exposed to a high dose of $1,25(OH)_2D_3$ and allowed to incubate without the metabolite. It would be of interest to determine if other activities, such as collagen synthesis and mineralization, are also increased in these bones.

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