Localization of 1,25-(OH)_2D_3-responsive Alkaline Phosphatase in Osteoblast-like Cells (ROS 17/2.8, MG 63, and MC 3T3) and Growth Cartilage Cells in Culture*

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Previous studies have shown 1,25-dihydroxyvitamin D_3 (1,25-(OH)_2D_3)-responsive alkaline phosphatase in cultured growth zone cartilage chondrocytes is localized in extracellular matrix vesicles (MV). Since osteoblast-like cells also have 1,25-(OH)_2D_3-responsive alkaline phosphatase, this study determined whether the 1,25-(OH)_2D_3-responsive enzyme activity is localized to MV produced by these cells as well. Osteoblast-like cells from rat (ROS 17/2.8), mouse (MC 3T3), human (MG 63), and rat growth zone cartilage were cultured in Dulbecco's modified Eagle's medium containing 10^{-7} - 10^{-14} M 1,25-(OH)_2D_3. Alkaline phosphatase total activity and specific activity were measured in the cell layer, MV, and plasma membrane (PM) fractions. MV and PM purity were verified by electron microscopy and MV alkaline phosphatase specific activity compared to PM (MV versus PM: ROS 17/2.8 6 x; MG 63, 5.5 x; MC 3T3, 33 x; GC, 2 x). There was a dose-dependent stimulation of MV alkaline phosphatase (5- to 15-fold increase at 10^{-7} - 10^{-8} M) in all cell types in response to the 1,25-(OH)_2D_3. The PM enzyme was stimulated in a parallel fashion in the osteoblast cultures. No effect of 1,25-(OH)_2D_3 was observed in growth cartilage PM. Although MV accounted for < 20% of the total activity they contributed 50% of the increase in alkaline phosphatase activity in the cell layer in response to 1,25-(OH)_2D_3 and MV specific activity was enriched 10 times over that of the cell layer. These are common features of MV produced by cells which calcify their matrix and suggest that hormonal regulation of MV enzymes may be important in primary calcification.

Alkaline phosphatase is clinically important in the mineralization of skeletal tissues (1) and is present in high levels in hypertrophic growth plate cartilage (2). There is a close association between this enzyme and in vivo calcification of cartilage (3, 4). Numerous studies indicate that mineral deposits in calcifying cartilage are first seen in alkaline phosphatase-enriched extracellular matrix vesicles which are derived from the chondrocyte plasma membrane (5-16). Several laboratories have presented data suggesting that alkaline phosphatase is also involved in matrix vesicle-mediated mineralization in vitro (17-20).

Alkaline phosphatase activity appears to be regulated by 1,25-dihydroxyvitamin D_3 (1,25-(OH)_2D_3).1 In vitamin D_3 deficiency, bone matrix synthesis and cartilage growth are inhibited, linear growth arrested, and bone formation retarded (21, 22). Treatment with vitamin D_3 restores mineralization and bone and cartilage formation. A direct effect of 1,25-(OH)_2D_3 on alkaline phosphatase has been noted in cultures of osteosarcoma cells (23-25), osteoblasts (26, 27), and chondrocytes (28, 29).

Matrix vesicle and plasma membrane enzyme activity in chondrocyte cultures are differentially regulated by 1,25-(OH)_2D_3 and 24,25-(OH)_2D_3 (30-31). Alkaline phosphatase in matrix vesicles produced by resting zone cells, which do not calcify their matrix in vivo, is stimulated by 24,25-(OH)_2D_3. In contrast, alkaline phosphatase in matrix vesicles produced by growth zone chondrocytes is stimulated by 1,25-(OH)_2D_3. These observations suggest that cells which calcify their matrix in vivo produce alkaline phosphatase activity in cultures that is 1,25-(OH)_2D_3-responsive.

Recently, studies were reported (32, 33) which demonstrated that osteoblasts derived from fetal rat and mouse calvaria produce matrix vesicles in culture. When these cells were incubated with β-glycerophosphate, crystals were observed in association with the matrix vesicles. Since alkaline phosphatase and vitamin D_3 have been implicated in hydroxyapatite formation, it was important to determine whether this enzyme is enriched in matrix vesicles produced by bone cells in culture and whether it is regulated by 1,25-(OH)_2D_3. Accordingly, cells from two tissues, calcifying cartilage and bone, and three species (rat, mouse, and human) were examined.

EXPERIMENTAL PROCEDURES

Chondrocyte Cultures—Growth zone chondrocyte cultures were used as controls in this study since we had shown previously that they produced matrix vesicles in culture which contain 1,25-(OH)_2D_3-responsive alkaline phosphatase activity (31). The system utilized has been described in detail by Boyan et al. (29). The costochondral growth zone cartilage was obtained by sharp dissection from the rib cages of 125-g Sprague-Dawley rats (30 rats/12 rats/experiment), separated, sliced, and incubated overnight in Dulbecco's modified Eagle's medium (DMEM) with 5% CO_2 in air at 37 °C. Chondrocytes were released from the cartilagenous matrix by sequential incubations in 1% trypsin (GIBCO) for 1 h and 0.02% collagenase (Worthington Type II) for 3 h. Enzymes were prepared in Hanks' balanced salt solution. Digests were filtered (40 mesh nylon), and the cells were collected by centrifugation at 500 x g for 5 min, resuspended in

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1 The abbreviations used are: 1,25-(OH)_2D_3, 1,25-dihydroxyvitamin D_3; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.
DMEM, and plated at a density of 25,000 cells/cm². Cultures were incubated in DMEM containing 10% fetal bovine serum (FBS) and 50 µg/ml vitamin C in an atmosphere of 5% CO₂ in air at 37 °C and 100% humidity. Medium was changed after 24 h and then at 72 h intervals. At confluence (7–10 days) cells were subcultured using the same dilutions and techniques previously described and allowed to return to confluence. Confluent, third passage cultures were used for all subsequent studies used in this study appeared by transmission electron microscopy.

Enzyme Activity—Specific activities of alkaline phosphatase (orthophosphoric-monoester phosphohydrolase alkaline (EC 3.1.3.1)) was determined for both the cell layer and the isolated membrane fractions and measured as a function of p-nitrophenol hydrolysis from p-nitrophenylphosphate at pH 10.2 (45).

Enzyme activity in the chondrocyte cell layer was measured following the method of Hale et al. (28). Confluent, second passage cells were subcultured to 24-well culture dishes (Corning) using a plating density of 25,000 cells/cm². Hormone was added at confluency, and cultures were incubated an additional 24 h. At harvest the medium was decanted, and the cell layer was removed using a cell scraper. After centrifugation, the cell layer pellet was washed with phosphate-buffered saline and resuspended in 50 µl of deionized water plus 25 µl of 1% Triton X-100.

Alkaline phosphatase activity in the osteoblast cell layer was measured using the assay described by Majeska et al. (24) scaled down to 96-well microtiter plates. The assay was performed as described previously (42). At harvest, the media was aspirated, the cells washed twice with phosphate-buffered saline, and 100 µl of 0.05% (v/v) Triton X-100 was added to each well. The microtiter plate was freeze-thawed twice, and each well mixed with a Titre-tek pipettor. An additional 100 µl of 0.05% Triton X-100 was added to the ROS 17/2.8 samples. Approximately 10–20% of the total cell lysate was used for the protein determination in all three cell lines. Approximately, 5–10% was used for the detection of alkaline phosphatase activity in the ROS 17/2.8 cells, whereas 50% was used for the MC 3T3 and MG 63 cell lines.

Specific activity of alkaline phosphatase was measured as follows. A protein standard consisting of 1–4 µg of human immunoglobulin G (Bio-Rad)/100 µl/well was assayed in triplicate in a 96-well plate with Triton X-100 concentration taken into account. Bio-Rad protein reagent (40 µl/well) was added and each well immediately mixed with a Titre-tek pipettor. The plate was read at 600 nm on an EIA plate reader (M. A. Bioproducts) between 5 and 30 min after mixing. Alkaline phosphatase substrate and standard were made according to the protocol of Majeska et al. (24) with the following modifications. The standard, p-nitrophenol (Sigma) consisted of 2–14 µmol/100 µl/well in AMP buffer (aminomethylpropanol, 0.5 M 2-amino-2-(hydroxymethyl)-propan-1-ol, pH 10.5, and 5 mM MgCl₂), in triplicate in a 96-well microtiter plate. 100 µl of 0.5 N NaOH was added to terminate the reaction after 10–15 min for the ROS 17/2.8 cells and after 60–90 min for the MC 3T3 and MG 63 cells and the plates read at 410 nm. Alkaline phosphatase specific activity was calculated as nanomoles of P/N/µg of protein/min.

Nucleotidase (5’-ribonucleotide phosphohydrolase (EC 3.1.3.5)) was measured as [¹⁴C]adenosine released from [³²P]AMP (46). This enzyme served as an internal reference of the relative purity of the membrane preparations. To facilitate comparison with the chondrocyte cultures, all data are expressed as micromoles of P/µg of protein/min.

Statistical Analysis—All data are expressed as the mean ± S.E. of six sample cultures or six wells. Figures and tables contain data from representative experiments. All experiments were performed a minimum of three times. Significance between data points and controls was determined using a two-tailed Student’s t test using <0.05 confidence limits.

RESULTS

Cell Layer—Specific activity of alkaline phosphatase in the cell layer varied with the source of cells. Of the osteoblast cell lines, ROS 17/2.8 cells had the highest specific activity (2.7 ± 0.3), followed by MC 3T3 cells (0.8 ± 0.5) and MG 63 cells (0.2 ± 0.0).

Matrix Vesicle Purification—The matrix vesicle preparations used in this study appeared by transmission electron microscopy to be rich with matrix vesicles and relatively free...
of other cell and matrix components. Fig. 1 is an electron micrograph of the matrix vesicle pellet obtained from cultures of ROS 17/2.8 cells and is typical of all of the matrix vesicles isolated from cartilage and bone cell cultures in this study. The vesicles were 0.05–0.2 μm in diameter and contained granular amorphous material. No crystals were present in the matrix vesicles. Fig. 2 is an electron micrograph of the plasma membrane pellet obtained from cultures of ROS 17/2.8 cells and is typical of all the plasma membrane preparations isolated in this study. The membranes were free of mitochondria, nuclear material, and matrix vesicles.

Effect of 1,25-(OH)2D3 on Cell Number—Bone cell number was decreased by 1,25-(OH)2D3 (Table I). The effect of hormone was greatest in the MC 3T3 cultures and least in the ROS 17/2.8 cultures. The decrease was statistically significant in MG 63 cells and MC 3T3 cells at 10–10–9 M but was observed in ROS 17/2.8 cells at 10–11 M only. There was a similar effect of hormone on subconfluent cultures as well (data not shown). The decrease in cell number observed was not due to a toxic effect of hormone. The viability of cells treated with 10–9 or 10–10 M 1,25-(OH)2D3, as determined by trypan blue dye exclusion, was identical to, or not significantly different from, nontreated controls for all cell lines examined.

Because of the decrease in cell number, all analyses of enzymatic activity were calculated both with respect to cell number and as a function of the protein content of the specific fraction.

Distribution of Alkaline Phosphatase—Distribution of alkaline phosphatase activity in cultures of growth zone chondrocytes confirmed that reported previously (31). ROS 17/2.8 cells exhibited enrichment of alkaline phosphatase specific activity in the matrix vesicle pellet when compared to the cell layer (86-fold) or to the plasma membrane (6-fold) (Table II). The distribution of activity in the ROS 17/2.8 cells was similar to that seen in the mouse and human cell lines (data not shown). Although the percent recovery of total activity in the matrix vesicle and plasma membrane fractions was comparable to that of growth zone chondrocytes (31), specific activity was higher in the chondrocyte cultures and specific membrane fractions (cell layer, 24 X; matrix vesicles, 3 X; plasma membranes, 9 X).

Enrichment of specific activity of alkaline phosphatase in the matrix vesicles with respect to the cell layer was markedly greater in the bone cell cultures than in the chondrocytes (chondrocytes, 11 X; ROS 17/2.8, 86 X). Similar enrichment of alkaline phosphatase activity in the matrix vesicles produced by MC 3T3 cells (Table III) and MG 63 cells (Table IV) was observed, when compared to the cell layer (Table V).

### Table I

| Control | Cell number × 10⁶ |
|---------|------------------|
|         | MG 63 | MC 3T3 | ROS 17/2.8 |
| M       | 4.12 ± 0.28  | 18.60 ± 2.23 | 26.26 ± 1.83 |
| 10−10   | 4.16 ± 0.21  | 22.56 ± 3.38 | 27.50 ± 1.21 |
| 10−11   | 4.13 ± 0.23  | 16.87 ± 3.03 | 25.90 ± 1.44 |
| 10−10   | 3.94 ± 0.25  | 13.23 ± 0.61 | 28.95 ± 1.55 |
| 10−9    | 2.37 ± 0.14* | 6.59 ± 0.40* | 24.96 ± 1.34 |
| 10−8    | 2.30 ± 0.19* | 3.12 ± 0.28* | 21.01 ± 1.46 |
| 10−7    | 2.26 ± 0.12* | 1.57 ± 0.14* | 16.38 ± 0.54* |

*Treatment versus control: p < 0.05.

### Table II

| Culture fraction | Specific activity | % recovery (fraction/total) |
|------------------|------------------|----------------------------|
|                  | μmol P/mg protein/min |                         |
| Cell layer       | 2.7 ± 0.3        |                           |
| Matrix vesicles  | 0.4 ± 0.1        |                           |
| 500 x g S         | 25.2 ± 8.0      | 13.3 ± 2.7                |
| 100,000 x g S     | 0.5 ± 0.0       | 4.6 ± 1.1                 |
| 100,000 x g MV    | 231.8 ± 20.7    | 15.7 ± 1.2                |
| Plasma membranes | 29.6 ± 3.5      |                           |
| 500 x g G          | 3.8 ± 1.1       | 10.0 ± 2.3                |
| 1,460 x g S       | 6.9 ± 1.4       | 12.0 ± 1.8                |
| 38,720 x g G      | 1.1 ± 0.4       | 10.9 ± 3.1                |
| 38,720 x g Mito'  | 6.7 ± 2.2       | 5.5 ± 1.1                 |
| 38,720 x g PM     | 38.4 ± 4.5      | 15.5 ± 1.9                |

* S, supernatant.  
* P, pellet.  
* Mito, mitochondria/membrane.
The specific activity of 5'-nucleotidase was also enriched in the matrix vesicles with respect to the plasma membranes in all osteoblast cell lines examined (Table VI). There was a 6.4-fold enrichment of this enzyme activity in matrix vesicles produced by MG 63 cells, a 4.3-fold enrichment in matrix vesicles produced by ROS 17/2.8 cells, and a 2.2-fold enrichment in matrix vesicles produced by MC 3T3 cells. Enzyme activity in the plasma membranes of all three osteoblast cell lines was essentially the same, averaging 148 cpm/mg protein/min.

Effect of 1,25-(OH)₂D₃ on Alkaline Phosphatase Activity in the Cell Layer—Stimulation of enzyme specific activity could be detected in the confluent ROS 17/2.8 cultures at 10⁻⁸-10⁻¹⁰ M with peak stimulation occurring at 10⁻⁸ M (Fig. 3). Although stimulation of enzyme activity in the subconfluent cultures was observed at the same concentrations of hormone, peak stimulation was at 10⁻⁶ M 1,25-(OH)₂D₃ (Fig. 4). In addition, the magnitude of response differed with respect to the degree of confluency at the time hormone was added to the cultures. Basal enzyme specific activity was two to three times higher in the confluent cultures. The subconfluent cultures incubated with 1,25-(OH)₂D₃ exhibited alkaline phosphatase activity...
comparable to that of the confluent control cultures. In contrast, alkaline phosphatase specific activity in the confluent cultures incubated with $10^{-7}$ M 1,25-(OH)$_2$D$_3$ was increased approximately 400%.

Although specific activity of alkaline phosphatase was significantly lower in the MG 63 and MC 3T3 cultures, both cell types showed a dose-dependent increase in response to hormone. The confluent MG 63 cells exhibited a statistically significant 2.5-fold increase in enzyme activity at $10^{-8}$ M 1,25-(OH)$_2$D$_3$. At $10^{-6}$ M, the increase was 12-fold. The increase in MC 3T3 alkaline phosphatase was significant at $10^{-7}$ M and was elevated 2.5-fold at $10^{-6}$ M 1,25-(OH)$_2$D$_3$. As was observed with ROS 17/2.8 cells, the basal activity of subconfluent cultures was lower, and at $10^{-7}$-$10^{-6}$ M 1,25-(OH)$_2$D$_3$, it approached that of the basal levels of the confluent cell layer (data not shown).

**Effect of 1,25-(OH)$_2$D$_3$ on Plasma Membrane and Matrix Vesicle Alkaline Phosphatase Activity**—Stimulation of total specific activity in the cell layer of all bone cells was observed at significantly lower sensitivities than were possible by measuring the isolated membrane fractions. Matrix vesicles isolated from confluent ROS 17/2.8 cultures exhibited a dose-dependent increase in enzyme activity which was statistically significant at $10^{-8}$-$10^{-7}$ M 1,25-(OH)$_2$D$_3$ (Fig. 5). The magnitude of response was independent of denominator, i.e. cell number or matrix vesicle protein content. Similarly, matrix vesicles from subconfluent cells exhibited a similar increase in specific activity, significant at $10^{-8}$ M (Fig. 6). The absolute levels of basal activity were comparable to those of matrix vesicles isolated from confluent cultures with respect to cell number, but the magnitude of stimulation was slightly lower in the subconfluent cultures at comparable hormone concentrations. Peak stimulation in confluent cultures was observed at $10^{-8}$ M 1,25-(OH)$_2$D$_3$, whereas enzyme activity had not plateaued in the subconfluent cultures at $10^{-7}$ M. When enzyme activity was calculated with respect to matrix vesicle protein content, basal levels in the subconfluent cultures were approximately one-half that of the confluent cultures. The fold stimulation was comparable, but peak levels were three times greater in matrix vesicles isolated from the confluent cells.

Basal levels of alkaline phosphatase in MC 3T3 cells were 0.8 ± 0.5 mmol of Pi/mg protein/min (Table V). Even at $10^{-7}$ M 1,25-(OH)$_2$D$_3$, the specific activity was only 1.7 ± 0.1. However, the basal level in the plasma membranes of these same cells was 0.9 ± 0.1 mmol of Pi/mg protein/min and at $10^{-7}$ M 1,25-(OH)$_2$D$_3$ was 2.0 ± 0.2 (Table III). The sensitivity afforded by measuring activity in the matrix vesicles was 33-fold greater than the plasma membranes and 37-fold greater than the cell layer. This sensitivity was evident in control cultures and in cultures incubated with 1,25-(OH)$_2$D$_3$. Enhancement of sensitivity was observed in matrix vesicles produced by MG 63 cells, although the magnitude of enhancement was not as great (Table IV).

In contrast to chondrocytes, 1,25-(OH)$_2$D$_3$ stimulated both the plasma membrane and matrix vesicle enzymes in bone cells. The magnitude of stimulation depended on the bone cell type and on the denominator. For instance, stimulation of matrix vesicle alkaline phosphatase was observed at $10^{-8}$ M 1,25-(OH)$_2$D$_3$ was 12.5 x in MC 3T3 cultures (Table III), 3.5 x in MG 63 cells (Table IV), and 2.2 x in ROS 17/2.8 cultures (Fig. 5), when data was calculated with respect to the cell number. In contrast, no differences in stimulation were detected when data were calculated as a function of matrix vesicle protein (MC 3T3, 2.1 x; MG 63, 2.0 x; ROS 17/2.8, 1.9 x). Stimulation of the plasma membrane enzyme at $10^{-8}$ M 1,25-(OH)$_2$D$_3$ was essentially the same for all three cell types and independent of denominator except for ROS 17/2.8 cells (micromoles of Pi/10$^6$ cells/min: MC 3T3, 3.3 x; MG 63,
DISCUSSION

Primary mineralization, characterized by the occurrence of extracellular matrix vesicles, has been described in various normal and pathologic tissues, including developing ephiphyseal cartilage, embryonic bone, bone wound repair, mantle dentin, osifying neoplasms, and ectopic calculations (9, 47–52). In calcifying tissues like growth cartilage and bone, mineralization is regulated by vitamin D and is associated with increased alkaline phosphatase activity, an enzyme that is enriched in the matrix vesicles isolated from cartilage. Although other papers have reported the influence of 1,25-(OH)2D3 on alkaline phosphatase in cell layers (24, 28, 42), there has been no effort to distinguish or pinpoint the actual location of the stimulated enzyme activity beyond release into culture media. Therefore, any effect of the vitamin D metabolite was ascribed to the cell population as a general phenomenon.

The current investigation has shown that the response to 1,25-(OH)2D3 is site-specific; the matrix vesicles are targeted and not the cell population per se. In the membrane and matrix vesicle preparations, half of total specific activity and most of the 1,25-(OH)2D3-responsive enzyme specific activity in osteoblast cultures is localized to the matrix vesicles. Furthermore, other investigators have utilized methodologies that separated the cell from the matrix prior to measurement of enzyme activity. Thus, only the cell population was examined for alkaline phosphatase activity, the matrix being discarded (25–27), therefore disregarding the significance of the alkaline phosphatase activity in the matrix and its regulation. This is no small oversight since recently matrix vesicles have been shown to mediate calcification by osteoblasts in vitro (32).

The matrix vesicles isolated in this study did not contain any evidence of crystals. Whether this was due to their loss during sample preparation or that they were not present in the cultures originally could not be determined under the experimental parameters used. Similar results have been obtained by other laboratories (14) as well as our own (29) using these procedures to isolate matrix vesicles from rat chondrocyte cultures. The matrix vesicles were also morphologically comparable to matrix vesicles observed in cartilage (9) and bone (53) in situ.

The data presented in this study support the theory that matrix vesicles are distinct organelles, although they are derived from the plasma membrane (54). For all osteoblast cell lines examined, the plasma membrane marker enzymes, alkaline phosphatase and 5'-nucleotidase, exhibited enriched activity in the matrix vesicle fraction. Previous observations in our laboratory (29) have shown that matrix vesicles produced by resting zone cells are also enriched in 5'-nucleotidase. Those produced by growth zone chondrocytes have elevated levels of this enzyme, but it is not statistically greater than that of the plasma membrane. Thus, specific enrichment of this enzyme does not appear to be a general characteristic of cells which calcify their matrix. Activity in the plasma membranes is comparable for all osteoepithel and is in the same order of magnitude as that of the growth zone chondrocytes (29). However, the fact that there is considerably more enrichment in the matrix vesicles produced by osteoblasts demonstrates fundamental biochemical differences in this organelle as it is produced by chondrocytes and osteoblasts. This observation is supported by the fact that the alkaline phosphatase: 5'-nucleotidase ratio varies between chondrocyte and osteoblast and among osteoblast cell lines.

The data presented in this paper indicate that the membrane isolation technique used yields those fractions of the culture which are most highly enriched in alkaline phosphatase specific activity, suggesting that enzyme present in the plasma membrane and matrix vesicles will be most responsive to metabolic regulation of the mineralization process. Since these two fractions represent only 34% of the total enzymatic activity, it is probable that subtle changes in alkaline phosphatase regulation might be missed if the entire culture were assayed. This is especially true for matrix vesicles which represent less than 20% of the total activity, yet are the sites most intimately involved in the mineralization process and the most highly enriched fraction in terms of specific activity.

Because 1,25-(OH)2D3 had an inhibitory effect on cell number, it was important to separate the influence of cell number from the influence of the amount of protein in a specific fraction in understanding the mechanism of hormone regulation. This is an important distinction. As demonstrated in Table III, the effect of 1,25-(OH)2D3 on matrix vesicles and plasma membranes isolated from MC 3T3 cells appears to be much greater when the data are expressed as a function of cell number rather than as specific activity of the isolated membrane fraction. Since the stimulation in the matrix vesicle specific activity is only 2-fold, the data may suggest that fewer cells are producing more vesicles per cell. Alternatively, the vesicles themselves may contain more total protein of which the stimulation in alkaline phosphatase is only 2-fold. Similar results were observed in cultures of MG 63 cells (Table IV) and in cultures of ROS 17/2.8 cells (Figs. 5 and 6). It is unlikely that the stimulation of alkaline phosphatase is merely a mathematical consequence of reduced cell number due to vitamin D toxicity, since the trypan blue dye exclusion studies demonstrated that the cells remained viable. Even though the cells were at confluence when hormone was added, they did continue to divide. This is a property of the chondrocytes and osteoblasts in cultures which we have noted routinely.

It was not surprising that matrix vesicles and plasma membranes isolated from the chondrocyte cultures respond so differently to 1,25-(OH)2D3. Although matrix vesicles are derived from the plasma membrane, they have a distinctly different biochemical composition. Both phospholipid composition (17, 29) and enzymatic activities (7, 30, 31) differ from those of the parent membrane. In fact, alkaline phosphatase is routinely enriched 2- to 3-fold in even crude matrix vesicle preparations (7) and, as a result, serves as the matrix vesicle marker enzyme. These observations suggest that matrix vesicle biogenesis and/or maturation yields a membrane capable of responding in a unique fashion to hormone signals. Since at least one function of matrix vesicles is to promote initial hydroxyapatite deposition (9), it may be that the action of vitamin D on these membranes in particular is to facilitate that process. This appears to be the case for bone cells as well. Difference in the behavior of the matrix vesicles isolated from the two cell types may be due to differences in their composition or in their regulation by the cell itself.

The results support the hypothesis that 1,25-(OH)2D3 may serve as a regulating influence on alkaline phosphatase specific to the matrix vesicle fraction (55). It is not known whether this regulatory effect is on the matrix vesicle in the matrix or on the cell membrane prior to the formation of the matrix vesicle. When data are normalized to cell number, the effect of hormone in bone cells is comparable in both fractions, suggesting that at least one regulatory mechanism is by incorporation of new enzyme into the plasma membrane and
subsequent release into the matrix as matrix vesicles, probably through activation of alkaline phosphatase gene transcription (56). The second level of regulation then occurs as a function of the differences in protein content (and composition) of the matrix vesicles and plasma membranes, resulting in a significant increase in specific activity in the matrix where mineralization is occurring. Although the rate of stimulation is the same in both membrane populations, the effect in the matrix is more activity.

The elevation of chondrocyte alkaline phosphatase activity by 1,25-(OH)$_2$D$_3$ observed in this study was parallel to that reported for cartilage slices in culture (57). Similarly, the stimulation of the osteoblast was comparable to that reported by other laboratories (23, 24, 34, 38, 41, 58). Recently reported increases in alkaline phosphatase activity in response to 1,25-(OH)$_2$D$_3$ in chondrocytes grown in serum-free media (28) might have been due to the response of these cells to an initial exposure to hormone. Initial exposure to hormone might also account for the reported physiologic concentration of vitamin D$_3$ at 10$^{-10}$ M. In the current study, chondrocyte and osteoblast cultures were acclimated to basal levels of hormone in the FBS-containing media; therefore, alkaline phosphatase activity was measured in response to additional hormone, and this may account for our observed physiologic concentration of hormone to be greatest at 10$^{-8}$ and 10$^{-7}$ M in confluent cells and 10$^{-6}$ M in subconfluent cells. Some of the differences in the magnitude of response noted for the chondrocytes and each of the osteoblast cell lines may have been a function of the differences in serum used in the media, resulting in varying endogenous hormone levels and, as a result, varying basal enzyme activity.

The degree of confluence is also a factor in interpreting the response of bone cells to hormone. Although stimulation of hormone is observed in both types of cultures, the magnitude is markedly different. The fact that subconfluent cultures incubated with hormone approximate basal levels of confluent cells may suggest that 1,25-(OH)$_2$D$_3$ is promoting cell differentiation at the expense of cell proliferation. This interpretation is supported by the observation that basal activities in matrix vesicles isolated from both cell cultures are comparable, especially when normalized to cell number. Differences in specific activity may be due to additional maturation of the matrix vesicles which occurs in confluent cultures only or because more mature vesicles are no longer being diluted by newly synthesized matrix vesicles in the subconfluent cultures. Thymidine incorporation studies (30) have demonstrated that cell proliferation is still occurring in the confluent cultures, although at much reduced rates, and this is further suppressed by exogenous hormone.

The subtle differences in osteoblast response would have been missed if stimulation of enzyme activity in the cell layer had been the sole parameter. For instance, in cultures of MC 3T3 cells, incubated with 10$^{-8}$ M 1,25-(OH)$_2$D$_3$, only a 25% increase in alkaline phosphatase specific activity was seen. There was a 60% increase in ROS 17/2.8 cells. Only in the MG 63 cells was a significant increase, 500%, observed at that hormone concentration. For all of the cell types, specific activity in the cell layer could be accounted for by that of the plasma membrane alone, thus obscuring the significant contribution of the matrix vesicle enzyme.

The data, therefore, indicate that the vitamin D$_3$ regulation of alkaline phosphatase at specific sites and in specific membrane populations, as first shown in rat costochondral growth zone chondrocytes, may be a generalized phenomenon unique to calcifying tissues. These results support the concept that not only in cartilage, but also in bone, primary mineralization is mediated by vitamin D$_3$ responsive matrix vesicles. These data are all the more significant in light of recent publications demonstrating that osteoblasts may utilize matrix vesicles as mediators of mineralization in vitro (32, 33). Extensive morphometric (59) and biochemical (60) studies using ablation of bone marrow to induce bone repair indicate that primary bone formation in vivo may be matrix vesicle-mediated as well.

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