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Regulation of arachidonic acid turnover by 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in growth zone and resting zone chondrocyte cultures

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Previous studies have shown that phospholipase A_2 activity in rat costochondral chondrocyte cultures is differentially regulated by 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ stimulates enzyme activity in growth zone chondrocytes but has no effect on the resting zone chondrocyte enzyme activity. 24,25-$(OH)_2D_3$ inhibits the resting zone enzyme but has no effect on the growth zone chondrocyte phospholipase A_2. This study examined whether the metabolites affect arachidonic acid turnover in their target cell populations. Incorporation and release of [^{14}C]arachidonate was measured at various times following addition of hormone to the cultures. Acylation and reacylation were measured independently by incubating half of the [^{14}C]arachidonate-labeled cultures with *p*-chloromercuribenzoate. The results demonstrated that the distribution of [^{14}C]arachidonate in membrane phospholipids differed between growth zone and resting zone chondrocytes and between the plasma membranes and matrix vesicles isolated from the growth zone chondrocyte cultures. Plasma membrane phospholipids were more susceptible to the release of [^{14}C]arachidonic acid by exogenous phospholipases than were matrix vesicle phospholipids. The effect of 1,25-$(OH)_2D_3$ on growth zone chondrocytes was observed within 5 min. Incorporation was greatest after 60 min; release was greatest after 30 min. 24,25-$(OH)_2D_3$ stimulated consistently elevated incorporation throughout the incubation period, peaking at 15 min. Peak release was at 60 min. The results confirm that resting zone chondrocytes and growth zone chondrocytes retain a differential phenotype in culture and demonstrate that matrix vesicles are distinct from the plasma membrane in terms of lipid composition and arachidonic acid incorporation. 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ appear to stimulate arachidonic acid turnover in their target cells by different mechanisms. Changes in fatty acid acylation and reacylation may be one mode of vitamin D-3 action in cartilage.

Introduction

The vitamin D metabolite, 1,25-dihydroxyvitamin D-3 (1,25-$(OH)_2D_3$), plays an active role in calcified cartilage differentiation and maturation [1]. Previous studies [2–6] indicate that cartilage cells have 24,25-dihydroxyvitamin D-3 (24,25-$(OH)_2D_3$) receptors, suggesting that they are responsive to this metabolite as well. Boyan et al. [7] have shown that chondrocytes at distinct stages of endochondral maturation respond differentially to these two vitamin D-3 metabolites. Cellular response includes changes in the activities of matrix vesicle enzymes like alkaline phosphatase [7] and phospholipase A_2 [8], matrix vesicle phospholipid composition [7], extracellular matrix protein synthesis and cell proliferation [9], and handling of calcium [10]. Specifically, 1,25-$(OH)_2D_3$ targets primarily growth zone cartilage cells and 24,25-$(OH)_2D_3$ the less differentiated resting zone cartilage cells.

Wuthier [11] has demonstrated that there is substantial phospholipase A_2 activity in mineralizing cartilage and has suggested that hydrolysis of lipids might be related to the calcification process. This hypothesis is supported by the fact that the activity of phospholipase A_2 is correlated with the differentiation of the epiphyseal growth plate. As chondrocytes hypertrophy and

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crystals begin to form in extracellular matrix vesicles, the activity of this enzyme is increased. Wuthier [12] has also shown that the phospholipid composition of matrix vesicles appears to be related to their functional role in mineralization. The increase in lysophospholipids is due in part to phospholipase A_2.

Recently, we demonstrated that the specific activity of phospholipase A_2 is enriched in matrix vesicles isolated from cultured rat costochondral chondrocytes [8]. Its activity can be differentially regulated by vitamin D-3 metabolites, depending on the metabolite used and the state of differentiation of the cell. For example, phospholipase A_2 is stimulated by 1,25-$(OH)_2D_3$ in matrix vesicles isolated from cultures of growth zone chondrocytes but there is no effect of 1,25-$(OH)_2D_3$ on the enzyme in matrix vesicles isolated from cultures of resting zone cells. In contrast, phospholipase A_2 is inhibited by 24,25-$(OH)_2D_3$ in matrix vesicles isolated from cultures of resting zone cells but this metabolite has no effect on phospholipase A_2 in matrix vesicles isolated from growth zone chondrocyte cultures [13]. Since differential regulation of phospholipase A_2 is also observed in isolated matrix vesicle membranes incubated directly with the vitamin D metabolites, that is in the absence of the chondrocyte and, therefore, of nuclear hormone receptors, gene transcription or new protein synthesis, it is likely that the effect of the hormone is directly on the membrane itself [8].

At least some of the differential response to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ may be due to a difference in the composition of the matrix vesicles [14]. Another possibility is that the basal metabolism of membrane phospholipids differs between the two membrane populations. Vitamin D-3 metabolites may regulate membrane enzyme activity and calcium transport across membranes, at least in part, by inducing a specific alteration in membrane phospholipids, leading to increased membrane fluidity [15,16]. One mechanism to accomplish this is by changing the activity of the resident phospholipases. This has been shown to be the case in intestinal brush border epithelium [17].

Studies by Matsumoto et al. [16] indicate that synthesis of phospholipids may also play a role. 1,25-$(OH)_2D_3$ enhanced the synthesis of phosphatidylcholine, independently of a protein synthetic pathway, and also enhanced the incorporation of unsaturated fatty acid, with resultant alterations in Ca^{2+} transport through the plasma membrane. These results have been substantiated by Levy et al. [18], showing that changes in phospholipid composition induced by 1,25-$(OH)_2D_3$ were related to changes in Ca^{2+} permeability.

Additional observations continue to strengthen the correlation between the action of vitamin D-3 metabolites and changes in membrane composition. For example, 1,25-$(OH)_2D_3$ induces mechanisms for the release of arachidonate from phospholipids [19] and 1,25-$(OH)_2D_3$ increases the activity of the intestinal phosphatidylcholine deacylation-reacylation cycle [20]. There is an accumulation of arachidonic acid in bone marrow-derived macrophages due to an increase in acyltransferase activity in response to this hormone [21].

A similar mechanism may be functional in cartilage as well. Therefore, in the current study we determined whether 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2H_3$ regulate the incorporation and release of [^{14}C]arachidonic acid in their respective target cell populations. An understanding of rapid, direct changes in fatty acid turnover stimulated by 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ may explain the mechanism of the direct action of vitamin D-3 metabolites in chondorcyte membranes. These studies also enabled us to test the hypothesis that there is active matrix vesicle phospholipid metabolism and that it differs with the state of chondrocyte differentiation.

Materials and Methods

Chondrocyte cultures. The culture system has been described in detail by Boyan et al. [13]. Rib cages were removed from 30 125-g Sprague-Dawley rats/experiment and placed in Dulbecco's modified Eagle's medium (DMEM). The resting zone and adjacent growth zone cartilage were separated, sliced, and incubated overnight in DMEM with 5% CO_2 in air at 37° C and 100% humidity. DMEM was replaced by two 20-min washes in Hank's balanced salt solution (HBSS), followed by sequential incubations in 1% trypsin for 1 h and 0.02% collagenase for 3 h. All enzymes were prepared in HBSS. After enzymatic digestion, cells were separated from debris by filtration, collected by centrifugation at $500 \times g$ for 10 min, resuspended in DMEM, and plated at a density of 10 000 cells/cm^2 for resting zone cells or 25 000 cells/cm^2 for growth zone cells. Cultures were incubated in DMEM containing 10% fetal bovine serum (FBS) and 50 μg/ml vitamin C in an atmosphere of 5% CO_2 in air at 37° C and 100% humidity. Media were changed at 24 h and then at 72-h intervals. At confluence (approximately 7–10 days), cells were subcultured using the same plating densities and technique as those described above and allowed to return to confluence. Fourth passage cells were used, since previous studies had shown that differential responses to vitamin D-3 metabolites are preserved at this passage (see Introduction above and Refs. 7–10, 13, 14, 22).

Preparation of cell fractions. Matrix vesicles and plasma membranes were prepared as described previously [14]. At harvest, cultures were trypsinized (1% in HBSS), the reaction was stopped with DMEM containing 10% FBS, and the cells were collected by centrifugation at $500 \times g$ for ten minutes, resuspended in 0.9% NaCl, washed twice, and counted. The trypsin digest supernatant was centrifuged for 10 min at $13\,000 \times g$ to

pellet a mitochondria/membrane fraction. The resultant supernatant was centrifuged for one hour at 100 000 × *g* to pellet matrix vesicles. The chondrocytes were homogenized and the plasma membranes were isolated by differential centrifugation, followed by sucrose density centrifugation. Membranes were resuspended as described below. All samples used in subsequent assays represent the combination of five cultures (i.e., five T-75 flasks).

Both the plasma membrane and matrix vesicle preparations contain plasma membrane marker enzymes (alkaline phosphatase, 5′-nucleotidase, ouabain-sensitive Na^+/K^+-ATPase, and phospholipase A_2) but they are differentially distributed between them [8,13]. Enrichment of alkaline phsphatase specific activity in the matrix vesicles is routinely 3–10-fold greater than in the plasma membrane. Previous studies [22] demonstrated that there is no contamination of the matrix vesicles or plasma membranes with mitochondrial enzymes.

Incorporation and release of arachidonic acid. [^{14}C]Arachidonic acid turnover was measured by the method of Kroner et al. [17]. [1-^{14}C]Arachidonic acid was obtained from New England Nuclear (Boston, MA) as an aqueous solution and diluted in DMEM so that 5 μl contained 0.05 μCi. Confluent, fourth passage growth zone and resting zone chondrocyte cultures were prelabeled by adding 5 μl [1-^{14}C]arachidonic acid (0.05 μCi) to the media. The cultures were incubated for 60 min and then washed three times with cold DMEM to stop the reaction. For studies assessing the distribution of [^{14}C]arachidonic acid in the matrix vesicle and plasma membrane phospholipids, membrane fractions were prepared (e.g., Table I).

For studies examining acylation and reacylation (e.g., Table II), cultures were incubated for an additional 60 min in 5 ml fresh DMEM containing 10% FBS. *p*-Chloromercuribenzoate (100 nmol/ml DMEM) (Sigma) was added to half of the cultures to block reacylation. All cultures were incubated an additional 60 min. At this time (time 0), the appropriate vitamin D-3 metabolite, either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, was added to the medium and the cells were cultured for six hours. The vitamin D-3 metabolites were solubilized in ethanol and these stock solutions were diluted at least 1 : 5000 (v/v) with DMEM prior to addition to the cultures [7]. Cultures with no exogenous hormone or vehicle and cultures incubated with ethanol at the same concentrations were used as internal controls: < 0.0005% ethanol for 1,25-$(OH)_2D_3$ and < 0.05% for 24,25-$(OH)_2D_3$. Both hormones were gifts of Dr. Milan Uskokovic of Hoffman-LaRoche (Nutley, NJ).

At 5, 15, 30, 60, 90, 120, 180, 270 and 360 min, 50 μl aliquots of culture media were collected and the content of [^{14}C]arachidonic acid determined. Reactions were stopped by addition of 5.0 ml chloroform/methanol (2 : 1, v/v) to each 50 μl aliquot. Phospholipids present in the resultant organic phase were separated by high-performance, thin-layer chromatography and ^{14}C content quantified by liquid scintillation spectroscopy. Phospholipids were identified by comparison of their R_f values with those of known standards (Sigma) run on the same plate. The percent release of [^{14}C]arachidonic acid was calculated as the amount of [^{14}C]arachidonic acid released to the media in the presence of *p*-chloromercuribenzoate at the specified time point, divided by the total [^{14}C]arachidonic acid in the cell layer and culture medium plus each 50 μl aliquot at the time of harvest at 360 min. The percent incorporation of [^{14}C]arachidonic acid was determined as the difference in the amount of [^{14}C]arachidonic acid released to the medium in the presence or absence of *p*-chloromercuribenzoate, at the specified time point, divided by the total [^{14}C]arachidonic acid in the cell layer and medium at harvest at 360 min. This experimental design [17] permits measurement of acylation and reacylation on the same population of cells. As a result, any incorporation of [^{14}C]arachidonate is reacylation since the only source of fatty acid is the pre-labeled cell itself.

In order to determine that the [^{14}C]arachidonate was actually incorporated into phospholipids, prelabeled membrane fractions were incubated with exogenous phospholipase C and phospholipase A_2 and the amount of [^{14}C]arachidonate released was determined. The effect of phospholipase C (EC 3.1.4.3) was measured as described by Low and Zilversmit [21]. The reaction mixture consisted of 15 μg phospholipase C (Sigma), 25 μg membrane protein (0.45 ml), and 2.35 ml 40 mM Tris acetate containing 0.002% NaN_3 (pH 7.8). Samples were incubated at 37 °C for 30 min. The incubation was stopped with the addition of 4 ml cold $CHCl_3/CH_3OH$ (2 : 1, v/v). Phospholipids were then identified as described below.

The action of phospholipase A_2 (EC 3.1.1.4) on the membrane lipids was measured as a function of hydrolysis of [^{14}C]arachidonate by a modification of techniques previously published [23]. Phospholipase A_2 (0.1 μg) (Sigma) and 0.5 ml of the membrane fraction containing 25 μg protein were added to 0.5 ml 100 mM Tris-HCl containing 2 mM $CaCl_2$ and 0.2 M NaCl. The mixture was incubated at 37 °C for 60 min and the reaction stopped by addition of 5 ml $CHCl_3/CH_3OH$ (2 : 1, v/v).

To eliminate the possibility that endogenous phospholipases might also be active or that a membrane component might inhibit the exogenous phospholipase, phospholipids were extracted in $CHCl_3/CH_3OH$ (2 : 1, v/v) and incubated directly with phospholipase A_2 as described by Christiansen et al. [24]. Samples (25 μl) were dried in microfuge tubes using N_2. 0.25 ml 10 mM Tris acetate (pH 7.0) was added to the dry film for 10 min at 35 °C. The tube was then sonicated for 5 min (two times) followed by addition of 5 μl phospholipase

A_2 (8 U, Sigma) for 15 min at 30 °C. The reaction was stopped with the addition of 5 ml $CHCl_3/CH_3OH$ (2 : 1, v/v).

Phospholipid analyses. Following extraction of the reaction mixtures with $CHCl_2/CH_2OH$ (2 : 1, v/v), the resultant organic phases were concentrated under N_2 and redissolved in 1 ml $CHCl_3/CH_3OH$ (2 : 1, v/v) containing 0.05% butylated hydroxytoluene (BHT). The extracts were analyzed by one-dimensional, high-performance thin-layer chromatography (HPTLC) [25]. Samples were separated on preformed silica gel G HPTLC plates (Analtech, Newark, DE). Plates were prewashed with $CHCl_3/CH_3OH$ (1 : 1, v/v), followed by ethanol/chloroform/ammonium hydroxide (50 : 6 : 6, v/v), also used as the mobile phase.

Phospholipids separated by HPTLC were visualized by heating. Each experimental HPTLC plate also contained a combined standard of 1 μg each bovine brain L-α-phosphatidylserine (PS), L-α-phosphatidylinositol (PI), cardiolipin (CL), L-α-phosphatidylcholine (PC) and sphingomyelin (Sph) and *E. coli* L-α-phosphatidylethanolamine (PE). All standards were obtained from Sigma Chemical Company (St. Louis, MO) and shown to be pure by HPLC (data not shown). Material was removed from the thin-layer chromatogram and counted by liquid scintillation spectroscopy.

Statistical analysis. Data on the distribution of [^{14}C]arachidonic acid in matrix vesicles and plasma membranes are expressed as the mean ± S.E. of six samples, where each sample represents the combined membranes from five T-75 flasks. Data on arachidonic acid turnover are expressed as the mean ± S.E. of six individual cultures. Significance between data points and controls was determined by Student's *t*-test using $P < 0.05$ confidence limits. Treatment/control ratios represent the means of six replicate experiments.

Results

The results of this study demonstrate that the distribution of ^{14}C-label in membrane phospholipids differs between growth zone and resting zone chondrocytes following a 60 min incubation in medium containing [^{14}C]arachidonic acid (Table I). For example, in matrix vesicles produced by growth zone chondrocytes, 2.4 times more [^{14}C]arachidonate is incorporated into sphingomyelin, but only half as much is incorporated into phosphatidylcholine or phosphatidylinositol as in matrix vesicles produced by resting zone cells. In contrast, incorporation of [^{14}C]arachidonate into phosphatidylserine was essentially the same for both cell types. These data were not compared statistically because different cell types were used and the cells were not harvested on the same day since they reached confluence at different times. However, the observations were consistent from experiment to experiment.

TABLE I

The incorporation of [^{14}C]arachidonate into phospholipids from membrane fractions isolated from confluent, fourth passage cultures of resting zone and growth zone chondrocytes

Each point is the mean ± S.E. of six samples. Data presented are from one representative experiment. Each experiment was repeated six times for growth zone chondrocytes (GC) and three times for resting zone chondrocytes (RC) with comparable results. Phospholipid standards were sphingomyelin (Sph), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and cardiolipin plus phosphatidylethanolamine (CL + PE). * Significant differences, $P < 0.05$, for matrix vesicles (MV) vs. plasma membranes (PM).

Phospholipid	% of total [^{14}C]arachidonic acid incorporated			
	GC		RC	
	MV	PM	MV	PM
Sph	34 ± 10	37 ± 1	14 ± 3	22 ± 3
PC	12 ± 0	21 ± 1 *	24 ± 1	31 ± 1 *
PI	11 ± 2	13 ± 1	21 ± 1	15 ± 3
PS	11 ± 2	9 ± 1	16 ± 1	26 ± 2 *
CL + PE	31 ± 6	20 ± 0 *	24 ± 2	5 ± 1 *

Distribution of [^{14}C]arachidonate also showed statistically significant variations between plasma membranes and matrix vesicles isolated from growth zone chondrocytes. Although radiolabeled fatty acid was present in comparable amounts in sphingomyelin and phosphatidylinositol, matrix vesicle phosphatidylcholine contained only half as much [^{14}C]arachidonate as did phosphatidylcholine in the plasma membranes of growth zone chondrocytes. In contrast, radiolabel was enriched in the phosphatidylserine and cardiolipin + phosphatidylethanolamine pools of the matrix vesicles. The cardiolipin + phosphatidylethanolamine pool also contains any free radio-labeled fatty acid.

Statistically significant differences in phospholipid [^{14}C]arachidonate content were observed in matrix vesicles and plasma membranes isolated from resting zone cell cultures as well (Table I). The plasma membrane contained 1.5-fold more radiolabeled sphingomyelin, phosphatidylcholine and phosphatidylserine than the matrix vesicles. In contrast, matrix vesicles contained slightly higher levels of radiolabeled phosphatidylinositol (not significant), but were significantly enriched in [^{14}C]arachidonate-labeled cardiolipin + phosphatidylethanolamine pool.

Following a second 60 min incubation in fresh DMEM (Table II), there were changes in the distribution of radiolabel in the matrix vesicle and plasma membrane phospholipids when compared to data presented in Table I (I: GC MV and PM vs. II: GC MV– and PM–). For example, the amount of [^{14}C]arachidonate incorporated into matrix vesicle phosphatidylcholine was increased. As a result, the differential distribution of [^{14}C]arachidonate between matrix vesicle and

TABLE II

The effect of p-chloromercuribenzoate on the incorporation of [^{14}C]arachidonate into matrix vesicles and plasma membranes isolated from growth zone chondrocyte cultures

Each point is the mean ± S.E. of six samples. Data presented are from one representative experiment. Each experiment was repeated four times with comparable results. Chondrocytes were incubated with (+) or without (−) *p*-chloromercuribenzoate. Phospholipid standards were sphingomyelin (Sph), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and cardiolipin plus phosphatidylethanolamine (CL + PE). No statistically significant differences were noted as a result of *p*-chloromercuribenzoate treatment or between matrix vesicles (MV) and plasma membranes (PM) from the same cultures.

Phospholipid	Percent of total [^{14}C]arachidonic acid incorporated			
	MV+	MV−	PM+	PM−
Sph	32±3	27±2	26±2	26±2
PC	20±2	20±1	16±3	18±1
PI	14±1	15±1	16±1	15±2
PS	8±1	9±1	11±1	13±3
CL + PE	26±2	35±4	33±1	27±2

plasma membrane phospholipids was lost. There was no significant effect of *p*-chloromercuribenzoate on the distribution of label in the phospholipid pools of either matrix vesicles or plasma membranes (Table II). This indicated that this reacylation blocking agent could be used without causing artifactual shifts in the [^{14}C]arachidonate incorporation measured. It should be stressed that the protocol which generated the data used in Table I differed from that used for data in Table II, with attendant differences in results. Both types of experiments were repeated multiple times and the data in each table, while from single experiments, are typical for their respective experimental protocol.

Reduction or loss of radiolabel from the phospholipid groups following incubation of the matrix vesicles or plasma membranes with either phospholipase C or phospholipase A_2 demonstrated that most of the [^{14}C]arachidonate had been incorporated into the phospholipid pool (Fig. 1). Loss of label was distributed evenly among the phospholipid classes, including sphingomyelin, phosphatidylcholine, phosphatidylinositol, and phosphatidylserine. Since cardiolipin and phosphatidylethanolamine run near the solvent front, along with any free fatty acids, any effect of either enzyme on them is unknown. As shown in Fig. 1, most of the released label was found in this region of the chromatogram.

Release of [^{14}C]arachidonic acid from the plasma membranes by exogenous phospholipases was distinctly different from its release from the matrix vesicle phospholipids (Fig. 1). Even after incubation with enzyme, between 15–76% of the label was retained by the matrix vesicle phospholipids. In contrast, virtually all of the label was released by enzymatic action from plasma membrane phospholipids. There were no differences noted between resting zone or growth zone chondrocyte cultures. Release of [^{14}C]arachidonic acid from isolated phospholipids followed the same pattern following incubation with phospholipase A_2 that was observed when the parent membranes were incubated with exogenous enzyme (Fig. 1).

Incubation of growth zone chondorcytes with 1,25-$(OH)_2D_3$ had a marked effect on incorporation and release of [^{14}C]arachidonic acid (Table III, Fig. 2). Examination of the treatment/control ratios presented in Fig. 2 demonstrate that the effects of hormone were observed within 5 min. In cultures incubated with 10^{-8} M 1,25-$(OH)_2D_3$, incorporation of [^{14}C]arachidonate was stimulated 2.2-fold at 5 min whereas release was stimulated only 1.3-fold. Incorporation peaked at 60 min whereas release peaked at 30 min and then returned to control levels by 360 min. Incubation of the growth

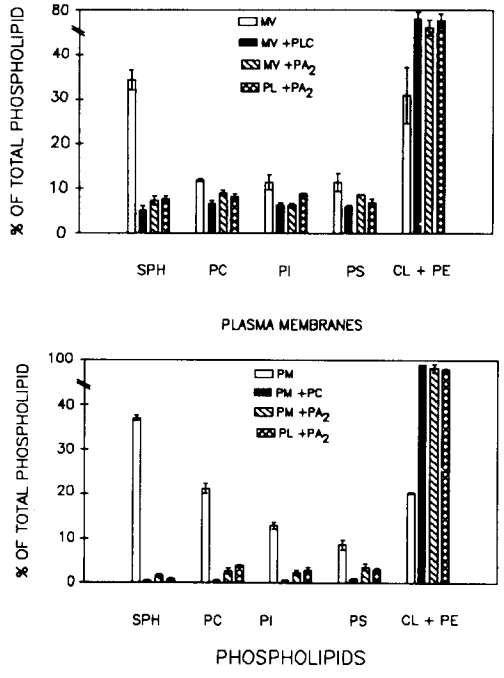

Fig. 1. Distribution of [^{14}C]arachidonic acid in phospholipids extracted from matrix vesicles and plasma membranes isolated from confluent, fourth passage growth zone costochondral chondrocytes. Pre-labeled plasma membranes (PM) or matrix vesicles (MV) were incubated with phospholipase C (PLC) or phospholipase A_2 (PA_2). Alternatively, phospholipids (PL) were extracted from the membranes and incubated with PA_2. Phospholipids were separated by high performance, thin-layer chromatography and identified by comparison to known standards: sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin + phosphatidylethanolamine (CL + PE). Values represent means ± S.E. for six samples. Data are from a single representative experiment. Each experiment was repeated three times with comparable results. All experimental values are statistically significant ($P < 0.05$) with respect to controls.

TABLE III

The effect of 1,25-$(OH)_2D_3$ on [^{14}C]arachidonic acid turnover by confluent, fourth passage growth zone chondrocytes in culture

* Sample vs. control: $P < 0.05$. Data are from one representative experiment of three replicate experiments, all yielding comparable results. Each value is the mean ± S.E. of four samples.

1,25-$(OH)_2D_3$	Incubation time (min)		
	30	90	270
	% [^{14}C]Arachidonic acid released		
0	37 ± 5	65 ± 8	75 ± 11
10^{-10} M	38 ± 7	69 ± 7	76 ± 5
10^{-9} M	59 ± 6 *	83 ± 8	85 ± 7
10^{-8} M	73 ± 10 *	86 ± 3 *	90 ± 3
	% [^{14}C]Arachidonic acid incorporated		
0	16 ± 3	27 ± 5	40 ± 12
10^{-10} M	19 ± 6	21 ± 11	45 ± 12
10^{-9} M	32 ± 14	26 ± 8	32 ± 9
10^{-8} M	43 ± 9 *	52 ± 4 *	59 ± 4

zone chondrocytes with 10^{-9} M 1,25-$(OH)_2D_3$ resulted in a similar response but to a much lesser magnitude, and incubation with 10^{-10} M 1,25-$(OH)_2D_3$ had no

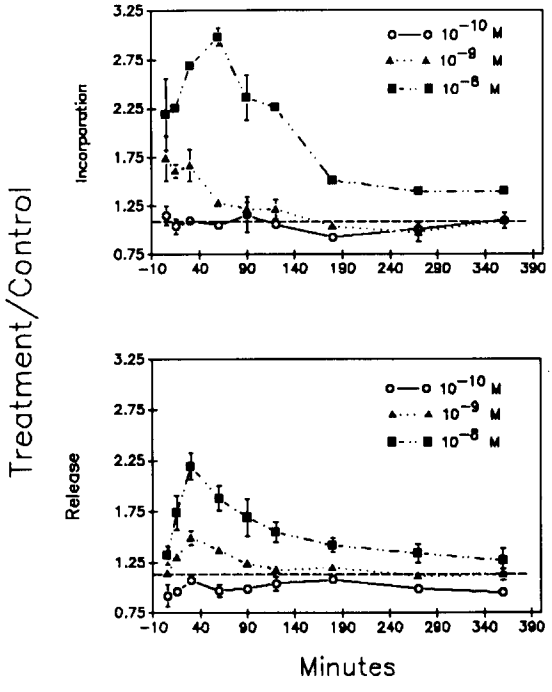

Fig. 2. Effect of 1,25-$(OH)_2D_3$ on [^{14}C]arachidonic acid turnover in confluent, fourth passage cultures of growth zone costochondral chondrocytes. Data are expressed as treatment/control ratios. Each value represents the means ± S.E. for six samples. Data are from a single representative experiment. Each experiment was repeated three times with comparable results. Data from one replicative experiment are presented in Table III. The following values are statistically significant ($P < 0.05$) with respect to controls. Incorporation: 10^{-9} M, 15 to 90 min; 10^{-8} M, 5 to 360 min. Release: 10^{-9} M, 15 to 180 min; 10^{-8} M, 5 to 270 min.

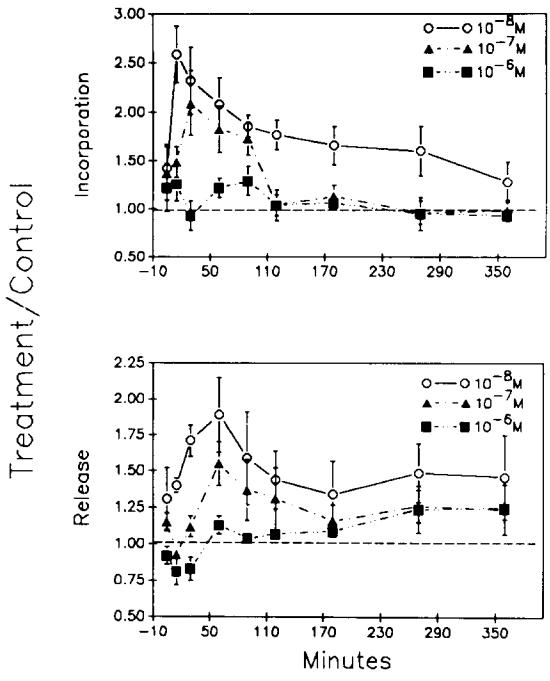

Fig. 3. Effect of 24,25-$(OH)_2D_3$ on [^{14}C]arachidonic acid turnover in confluent, fourth passage cultures of resting zone costochondral chondrocytes. Data are expressed as treatment/control ratios. Each value represents the mean ± S.E. for six samples. Data are from a single representative experiment. Each experiment was repeated three times with comparable results. Data from one replicative experiment are presented in Table IV. The following values are statistically significant ($P < 0.05$) with respect to controls. Incorporation: 10^{-7} M, 15 to 90 min; 10^{-8} M, 15 to 270 min. Release: 10^{-7} M, 60 min; 10^{-8} M, 15 to 90 min.

effect on either parameter. The statistical significance of the data are indicated in the figure legend.

When data are expressed as treatment/control ratios, it is clear that the relative effect of 1,25-$(OH)_2D_3$ was greater on [^{14}C]arachidonate incorporation when compared to release. However, examination of the actual data obtained in a comparable experiment (Table III) demonstrates that in absolute amounts, more label was released than was incorporated. For example, in the control cultures, the percent incorporation of [^{14}C]arachidonate was 16.4% at 30 min whereas the percent release of labeled material was 37%. Even after 270 min, the amount of label released (75%) was two times greater than the amount that was incorporated (40%).

Incubation of resting zone cells with 24,25-$(OH)_2D_3$ also affected incorporation of [^{14}C]arachidonate (Fig. 3). Cells exposed to 10^{-8} M 24,25-$(OH)_2D_3$ exhibited consistently elevated incorporation of label throughout the incubation period. Peak stimulation (2.5-fold) was seen at 15 min. At 10^{-7} M 24,25-$(OH)_2D_3$ the effect peaked at 30 min, but was over by 120 min. There was essentially no effect of hormone at 10^{-6} M. Statistical significance of the data are indicated in the figure legend.

TABLE IV

The effect of 24,25-$(OH)_2D_3$ on [^{14}C]arachidonic acid turnover by confluent, fourth passage resting zone chondrocyte cultures

* Sample vs. control: $P < 0.05$. Data are from one representative experiment of three replicate experiments, all yielding comparable data. Each value is the mean ± S.E. of four samples.

24,25-$(OH)_2D_3$	Incubation time (min)		
	30	90	270
	% [^{14}C]Arachidonic acid released		
0	27 ± 5	25 ± 4	28 ± 5
10^{-8} M	51 ± 1 *	70 ± 4 *	76 ± 3 *
10^{-7} M	31 ± 6	42 ± 5 *	52 ± 5 *
10^{-6} M	21 ± 2	25 ± 2	41 ± 4
	% [^{14}C]Arachidonic acid incorporated		
0	5 ± 2	13 ± 2	13 ± 2
10^{-8} M	34 ± 6 *	51 ± 7 *	54 ± 3 *
10^{-7} M	10 ± 3	17 ± 3	19 ± 4
10^{-6} M	3 ± 1	15 ± 3	19 ± 3

Release of [^{14}C]arachidonate in resting zone cells was also stimulated by 24,25-$(OH)_2D_3$ (Fig. 3). Peak stimulation (1.8-fold) of release was seen in cells incubated with 10^{-8} M hormone at 60 min. The magnitude of stimulation was reduced in cells incubated with 10^{-7} M 24,25-$(OH)_2D_3$ and was not significant in cells incubated with 10^{-6} M hormone. As shown in Table IV (data are derived from a comparable experiment to those in Fig. 3), in absolute values, the amount of radiolabel released was greater than the amount incorporated for unstimulated cells as well as for the experimental cultures.

Discussion

Our data support the hypothesis that vitamin D-3 metabolites regulate membrane function by changing the composition of membrane phospholipids [17,26,27] via restructuring of their fatty acid moieties. There is mounting evidence to support this hypothesis. For example, Matsumoto et al. [16] demonstrated that 1,25-$(OH)_2D_3$ stimulated synthesis of phosphatidylcholine and elevated the incorporation of unsaturated fatty acids. Others [28] have reported that 1-$(OH)D_3$ caused a change in the fatty acid composition of phosphatidylcholine in chick intestinal microvillus membranes.

As in the present study, both deacylation and reacylation can be affected, thereby serving as a point for regulation. 1,25-$(OH)_2D_3$ has been shown to induce mechanisms for the release of arachidonate from phospholipids [19]. Changes in the deacylation-reacylation cycle can alter significantly membrane parameters such as fluidity and even permeability and transport [20].

Whether the effect of 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ on arachidonic acid turnover is cell-specific was not examined. Previous studies had shown that stimulation or inhibition of phospholipase A_2 activity by 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ was dependent on the state of chondrocyte differentiation [8]. Nonetheless, the results indicate that each metabolite affected its target cell population in a different manner. This may have been due to differences in the basal phospholipid metabolism of the cell or to structural differences in the membranes.

The net effect of both metabolites on their respective target cells is to stimulate arachidonic acid release. Other investigators [19] have shown that cells can respond to 1,25-$(OH)_2D_3$ by increasing arachidonic acid release. Variations in arachidonic acid content and turnover, like those observed in the present study, might effect a wide range of cellular and membrane events [29,30] with important implications for the mechanism of vitamin D action. For example 1,25-$(OH)_2D_3$-differentiated HL-60 cells further metabolize [^{14}C]arachidonate to cyclooxygenase and lipoxygenase products, suggesting that these compounds may play a role in autocrine or paracrine regulation [19]. Low doses of prostaglandin E_2 are known to stimulate bone formation [31–34] and bone cell activity [25,36]. Ca ion flux may also be regulated since prostaglandin E_2 is an effective activator of the phosphoinositol pathway in cultured osteoblasts [36]. However, the effects of prostaglandins and leukotrienes on chondrocytes have not been investigated in the chondrocyte culture system used in this study.

Independent of target cell or metabolite, there appears to be two effects of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_3D_3$, one that is short term and one that is long term. When chondrocyte cultures were incubated for 24 h with hormone [8], 1,25-$(OH)_2D_3$ stimulated phospholipase A_2 activity in the growth zone cell cultures whereas 24,25-$(OH)_2D_3$ inhibited phospholipase A_2 activity in the resting zone cultures. In the short term cultures used in the present study, both acylation and reacylation were stimulated in each cell by its respective metabolite. However, in resting zone cells incubated with 10^{-6} M 24,25-$(OH)_2D_3$, there was a transient inhibition in arachidonic acid release. It is important to note that the earlier study [8] only addressed the activity of endogenous phospholipase A_2 on exogenous phosphatidylethanolamine while the present study measured total release of [^{14}C]arachidonate from pre-labeled phospholipids in the culture.

The data indicate that there is active phospholipid metabolism in matrix vesicles that is independent of the cell. Not only are the matrix vesicle phospholipids labeled differently from those of their respective parent plasma membranes but there are distinct differences between the cell types, confirming our previous observations that the chondrocytes retain their differential phenotype in culture [7–10,13,14,22]. While our previous studies demonstrated that there is resident phospho-

lipase A_2 activity in the matrix vesicles [13], the present studies demonstrate that the matrix vesicles can also reacylate their phospholipids. Only arachidonic acid metabolism was examined; there may be other differences in fatty acid turnover as well.

Although the phospholipid composition of matrix vesicles produced by the growth zone chondrocytes is enriched in phosphatidylserine and sphingomyelin in comparison to the plasma membrane [13,26,37], the percent of [^{14}C]arachidonic acid incorporated into these phospholipids was the same in both membrane fractions, indicating that not all of each phospholipid was labeled. In addition, three times more [^{14}C]arachidonic acid was incorporated into the sphingomyelin pool than was incorporated into the phosphatidylserine pool in both membrane fractions isolated from the growth zone chondrocytes. One explanation for this is that the phsophatidylserine was protected by the formation of calcium-phospholipid-phosphate complexes, which are present in matrix vesicles isolated from the hypertrophic zone of epiphyseal cartilage [38] and have been shown by Boskey (personal communication) to be resistant to phospholipase A_2 activity. It has been hypothesized that these complexes are formed on the plasma membrane prior to matrix vesicle production [20]. In contrast, there was no difference in [^{14}C]arachidonic acid incorporation in the sphingomyelin and phosphatidylserine pools of matrix vesicles or plasma membranes isolated from resting zone chondrocyte cultures which are derived from cartilage which does not mineralize its matrix or form calcium-phospholipid-phosphate complexes in vivo [39].

The relative resistance of matrix vesicle membranes and isolated phospholipids to enzyme action may relate to any of several factors. Phospholipase A_2 can also be inhibited by lyso-derivatives released from the membrane surface as a consequence of enzyme action, forming complexes with the enzyme [40]. Different phospholipid compositions would result in differential release of lyso-derivatives, reflected in the products released from the respective matrix vesicles or plasma membrane populations examined. Differences in matrix vesicle and plasma membrane conformation may play a role as well. For example, in mitochondria, the activity of phospholipase A_2 is greatly influenced by the phospholipid composition and structure, either bilayer or hexagonal H_{II} array, of the substrate aggregates [41]. The importance of substrate organization has also been demonstrated using phosphatidylcholines containing fatty acids of varying chain lengths [42].

Differences in composition and conformation may be of considerable importance in providing a suitable membrane environment for initial mineral formation. Phospholipase activity is postulated to be sterically inhibited by the presence of mineral associated with the proteolipids (Boyan, unpublished data) and calcium-phospholipid-phosphate complexes associated with initiation of mineralization [43]. At the same time, other matrix vesicle phospholipids, like phosphatidylethanolamine, are preferentially degraded [12].

Alterations in membrane phospholipids have the potential for altering calcium movements through membranes without synthesis of new proteins [18,38,44] and may be the mechanism by which 1,25-$(OH)_2D_3$ stimulates calcium movement through membranes. Our own studies [10] support this hypothesis, since resting zone cells and growth zone chondrocytes exhibit rapid changes in $^{45}Ca^{2+}$ flux in response to 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, respectively, suggesting that new protein synthesis has not yet occurred. Changes in $^{45}Ca^{2+}$ content of the chondrocytes and their matrix vesicles, as well as the hormone-dependent effects on alkaline phosphatase and phospholipase A_2 activities, cannot be mimicked by the Ca^{2+} ionophore, A23187 (data not shown), indicating that the effects of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ may involve mechanisms other than just calcium influx per se. Baran et al. [26], have shown that the effects of 1,25-$(OH)_2D_3$ on ctosolic calcium in hepatocytes are completely blocked by inhibitors of phospholipase A_2.

In summary, this study demonstrates that phospholipid metabolism in matrix vesicles, at least with respect to [^{14}C]arachidonic acid metabolism, is active and may be independent of the cell. The turnover of this fatty acid is regulated by vitamin D metabolites. The effects of 1,25-$(OH)_2D_3$ on its target cell population are different from the effects of 24,25-$(OH)_2D_3$ on its target cell population. Changes in arachidonic acid turnover may be one mechanism by which vitamin D metabolites elicit their effects on cells.

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References

1 Norman, A.W. (1980) Contrib. Nephrol. 18, 1–11.
2 Garabedian, M., DuBois, M.B., Corvol, M.T., Pezant, E. and Balsan, S. (1978) Endocrinology 102, 1262–1268.
3 Corvol, M., Ulmann, A. and Garabedian, M. (1980) FEBS Lett. 116, 273–276.
4 Binderman, I. and Somjen, D. (1984) Endocrinology 115, 430–432.
5 Fine, N., Binderman, I., Somjen, D., Earon, Y., Edelstein, S. and Weisman, Y. (1985) Bone 6, 99–104.
6 Somjen, D., Somjen, G., Harrell, A., Mechanic, G. and Binderman, I. (1982) Biochem. Biophys. Res. Commun. 106, 644–651.
7 Boyan, B.D., Schwartz, Z., Carnes, D.L. and Ramirez, V. (1988) Endocrinology 122, 2851–2860.

8 Schwartz, Z. and Boyan, B. (1988) Endocrinology 122, 2191–2198.
9 Schwartz, Z., Schlader, D., Ramirez, V., Kennedy, M. and Boyan, B. (1989) J. Bone Min. Res. 4, 199–207.
10 Langston, G.G., Swain, L.D., Schwartz, Z., del Toro, F., Gomez, R. and Boyan, B.D. (1990) Calcif. Tissue Int., in press.
11 Wuthier, R.E. (1973) Clin. Orthop. Rel. Res. 90, 191–200.
12 Wuthier, R.E. (1975) Biochim. Biophys. Acta 409, 128–143.
13 Schwartz, Z., Schlader, D., Swain, L. and Boyan, B. (1988) Endocrinology 123, 2878–2883.
14 Boyan, B.D., Schwartz, Z., Swain, L.D., Carnes, D.L., Jr. and Zislis, T. (1988) Bone 9, 185–194.
15 Rasmussen, H., Matsumoto, T., Fontaine, O. and Goodman, D.B. (1982) Fed. Proc. 41, 72–77.
16 Matsumoto, T., Fontaine, O. and Rasmussen, H. (1981) J. Biol. Chem. 256, 3354–3360.
17 Kroner, E.E., Peskar, B.A., Fischer, H. and Ferber, E. (1981) J. Biol. Chem. 256, 3690–3697.
18 Levy, R., Nathan, I. and Shany, S. (1987) Biochim. Biophys. Acta 902, 178–182.
19 Stenson, W.F., Teitelbaum, S.L. and Bar-Shavit, Z. (1988) J. Bone Min. Res. 3, 561–571.
20 O'Doherty, P.J. (1978) Lipids 14, 75–77.
21 Low, M.G. and Zilversmit, D.B. (1980) Biochim. Biophys. Acta 596, 223–234.
22 Schwartz, Z., Knight, G., Swain, L. and Boyan, B. (1988) J. Biol. Chem. 263, 6023–6026.
23 Colard-Torquebiau, O., Wolf, C. and Bereziat, G. (1986) Biochemistry 58, 587–592.
24 Christiansen, K., Demant, F.J.F. and Carlsen, J.S. (1985) Anal. Biochim. 145, 63–66.
25 Dugan, E.A. (1985) Liq. Chromatogr. 3, 126–128.
26 Baran, D.T., Sorensen, A.M. and Honeyman, T.W. (1988) J. Bone Min. Res. 3, 593–600.
27 Putkey, J.A., Nemere, I. and Norman, A.W. (1986) J. Bone Min. Res. 1, 305–311.
28 Rasmussen, H., Max, E.E. and Goodman, D.B.P. (1977) in Vitamin D. Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., Schaefer, K., Coburn, J.W., DeLuca, H.F., Fraser, D., Grigoleit, H.G. and Herrath, D.V., eds.), pp. 913–925, Walter de Gruyter, New York.
29 Schaffer, B.E. and Zadunaisky, T.A. (1979) Biochim. Biophys. Acta 556, 131–143.
30 Klausner, R.D., Kleinfeld, A.M., Hoover, R.L. and Karnovsky, M.T. (1980) J. Biol. Chem. 255, 126–1296.
31 Chyun, Y.S. and Raisz, L.G. (1984) Prostaglandins 27, 97–103.
32 Ueda, K., Saito, A., Nakano, H., Oashima, M., Yokota, M., Muraoka, R. and Iwaya, T. (1980) J. Pediatr. 97, 834–836.
33 Ringel, R.E., Brenner, J.I., Haney, P.J., Burns, J.E., Moulton, A.L. and Berman, M.A. (1982) Radiology 142, 657–658.
34 Lund, J.E., Brown, W.P. and Tregerman, L. (1982) in Prostaglandin in Clinical Medicine: Cardivascular and Thrombotic Disorders (Wu, K.K. and Rossi, E.C., eds.), pp. 93–109, Yearbook Medical Publisher, Inc., Chicago.
35 Yokota, K., Kusaka, M., Ohshima, T., Yamamoto, S., Kurihara, N., Yoshino, T. and Kumegawa, M. (1986) J. Biol. Chem. 261, 15410–15415.
36 Farndale, R.W., Sandy, R., Atkinson, S.J., Pennington, S.R., Meghji, S. and Meikle, C.M. (1988) Biochim. J. 252, 263–268.
37 Wuthier, R.E. (1976) Fed. Proc. 35, 117–121.
38 Wuthier, R.E. and Gore, S.T. (1977) Calcif. Tissue Res. 24, 163–171.
39 Boskey, A.L., Goldburg, M.R. and Posner, A.S. (1978) Proc. Soc. Exp. Biol. Med. 157, 590–593.
40 Kupferberg, J.P., Yokoyama, S. and Kezdy, F.J. (1981) J. Biol. Chem. 256, 6274–6281.
41 Lenting, H.B.M., Nicolay, K. and Van der Bosch, H. (1988) Biochim. Biophys. Acta 958, 405–415.
42 De Haas, G.H., Bonsen, P.P.M., Pieterson, W.A. and Van Deenen, L.L.M. (1971) Biochim. Biophys. Acta 239, 252–266.
43 Raggio, C.L., Boyan, B.D. and Boskey, A.L. (1986) J. Bone Min. Res. 1, 409–415.
44 Tanaka, Y. and DeLuca, H.F. (1971) Arch. Biochem. Biophys. 146, 574–578.