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1,25-Dihydroxyvitamin D-3 alters membrane phospholipid composition and enhances calcium efflux in HL-60 cells

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1,25-Dihydroxyvitamin D-3 ($1,25(OH)_2D_3$) had direct effects on the HL-60 cell membrane. Treatment of HL-60 cells with $1,25(OH)_2D_3$ for short time periods (2–4 hours) caused an increase in calcium efflux. This phenomenon was found to be unrelated to new protein synthesis since it was not inhibited in the presence of RNA and protein synthesis inhibitors. The treatment of the HL-60 cells with $1,25(OH)_2D_3$ for four hours caused changes in their membrane phospholipid composition. The phosphatidylcholine: phosphatidylethanolamine ratio increased from 1.2 to 1.5. Thus the alteration in the phospholipid composition in the membrane induced by $1,25(OH)_2D_3$ may be responsible for the changes in the permeability of the membrane to calcium ions.

Introduction

It has previously been shown that the active metabolite of vitamin D-3, 1,25-dihydroxyvitamin D-3 ($1,25(OH)_2D_3$), inhibits proliferation and induces differentiation of HL-60 cells to monocytes [1–4], although its mode of action is unknown. The main physiological effect of $1,25(OH)_2D_3$ is the control of calcium transport in cells. This was clearly established in the intestine, bone and kidney cells [5–7]. Rasmussen and colleagues [8–12] have suggested that $1,25(OH)_2D_3$ acts partially through direct effects on plasma membrane phospholipids which result in changes in membrane permeability to Ca^{2+} ions in intestine cells. They have suggested that the phospholipid effect may be an alternative to the classical scheme of steroid hormone action, i.e., steroid hormone receptor binding, genome activation and synthesis of specific proteins [13,14].

Many reactions taking place in cells are known to be influenced by Ca^{2+} ions and it is thus clear that the regulation of the intracellular concentration of Ca^{2+} is a phenomenon of paramount physiological significance. Changes in calcium transport through the cell membranes may thus play a role in regulating growth and differentiation processes.

This study suggests that there is a direct effect of $1,25(OH)_2D_3$ on the HL-60 membrane phospholipids which may affect the transport of calcium ions through the cells.

Materials and Methods

Unlabelled $1,25(OH)_2D_3$ and 25-hydroxyvitamin D-3 ($25(OH)D_3$) were kindly provided by Hoffmann-la Roche, Basel. The compound was reconstituted in ethanol and stored in concentrated solutions at −20°C. The vitamin D metabolites were freshly diluted in the appropriate

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medium before each experiment. The ethanol concentration in the test conditions and the controls never exceeded 0.1%. $^{45}Ca^{2+}$ (2 mCi/ml) was purchased from Amersham International plc, U.K.

Cell culture. The HL-60 cells were grown in stationary suspension culture in RPMI-1640 medium which contained 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 12.5 U/ml nystatin (Biological Industries, Beth Haemek, Israel) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Ca^{2+} efflux rates. Ca^{2+} efflux was measured by a modification of the method of Schimmel and Hallam [16]. Radiolabelled calcium ($^{45}Ca^{2+}$) (10–20 μCi/ml) was added to the HL-60 cells during the last hour of treatment with the vitamin D metabolites. The cells ($5 \cdot 10^6$) were washed three times at 4°C with phosphate-buffered saline (pH 7.4). They were incubated at 25°C in 2 ml RPMI medium (Ca-rich medium) and with 2 ml RPMI containing 2 mM EGTA (Ca-free medium). Aliquots were withdrawn at appropriate intervals and filtered through membrane filters (BA 84 0.3 μm, Tamar, Israel). The remaining radioactivity in the cells was determined by a Liquid Scintillation Spectrometer (Packard).

Membrane phospholipid determination. Plasma membranes were separated by differential centrifugation according to Stephen and Cooper [17]. The phospholipids were separated by two-dimensional thin-layer chromatography according to Yavin and Zutra [18]. The membranes were dissolved in chloroform/methanol/water (8:4:3, v/v/v). The upper phase was discarded and the lower chloroform phase containing the lipid extract was evaporated to dryness using nitrogen and then dissolved in 25 μl chloroform/methanol (2:1, v/v). The mixture for the first running direction was chloroform/methanol/40% methylamine (13:6:1.5, v/v/v). The mixture for the intermediary run was diethyl ether/acetic acid (glacial) (19:1, v/v) and for the second direction chloroform/ acetone/ methanol/ acetic acid/ water (10:4:2:3:1, by vol.). Phospholipids were identified by comparison with authentic reference compounds (Sigma). For quantitative determinations, the areas of silica gel representing the respective phospholipids were removed with a razor blade and transferred to tubes. The phosphate content of each spot was determined quantitatively [19].

Results

The short-term effect of the active metabolite of vitamin D-3 on Ca^{2+} efflux from HL-60 cells was studied. The cells were incubated with 10^{-7} M $1,25(OH)_2D_3$ for up to four hours and then the efflux of radioactive calcium ($^{45}Ca^{2+}$) into calcium-free medium was measured. As shown in Fig. 1, incubation with $1,25(OH)_2D_3$ for two hours and above caused an increase in the calcium efflux from the cells. The amount of $^{45}Ca^{2+}$ loaded was identical in the different treatments. The $^{45}Ca^{2+}$ efflux was expressed as percentage of total amount loaded. The differences in $^{45}Ca^{2+}$ between the control and the treated cells are indeed small but significant and reproducible.

The dose-response effect of $1,25(OH)_2D_3$ on the $^{45}Ca^{2+}$ efflux from HL-60 cells was studied. As shown in Fig. 2, the inhibition of $^{45}Ca^{2+}$ efflux by $1,25(OH)_2D_3$ is dose dependent from 10^{-10} to 10^{-7} M.

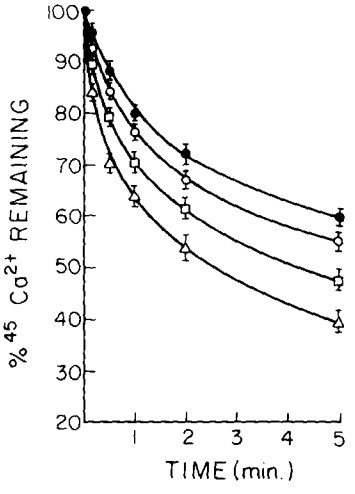

Fig. 1. Time dependence of $1,25(OH)_2D_3$ treatment on the $^{45}Ca^{2+}$ efflux from HL-60 cells. The cells were treated for up to 4 h with 10^{-7} M $1,25(OH)_2D_3$ and the efflux of $^{45}Ca^{2+}$ from the cells was measured for 5 min. Values are means ± S.E. of five experiments. Calcium-free medium contains 2 mM EGTA. Symbols: ●, control; treatment with $1,25(OH)_2D_3$ for: ○, 2 h; □, 3 h, and △, 4 h. Differences were found to be significant after 3 and 4 h ($P < 0.025$ and $P < 0.001$, respectively).

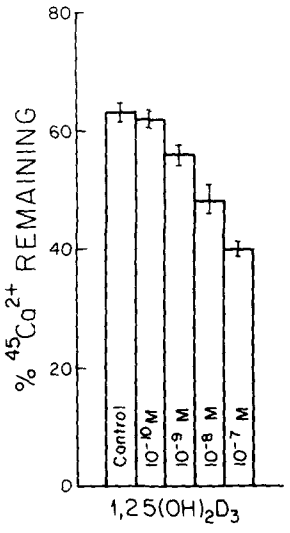

Fig. 2. Dose-dependent effect of $1,25(OH)_2D_3$ treatments on % $^{45}Ca^{2+}$ remaining in HL-60 cells. The $^{45}Ca^{2+}$ efflux was studied for 5 min. Values are means ± S.E. of three experiments.

Fig. 3 represents the efflux of $^{45}Ca^{2+}$ from HL-60 cells to calcium-free medium (A) and to calcium-rich medium (B) after treatment with vitamin D metabolites for four hours. While $1,25(OH)_2D_3$ caused an increase in Ca^{2+} efflux, $25(OH)D_3$ had no effect. The Ca^{2+} efflux into calcium-free medium was more affected by $1,25(OH)_2D_3$ than the calcium efflux into the calcium-rich medium. The effect of $1,25(OH)_2D_3$ on Ca^{2+} efflux was also evident in the presence of RNA and protein synthesis inhibitors (actinomycin D 0.24 nM and cycloheximide 210 nM, respectively), indicating that synthesis of new proteins is not involved in the process.

Inhibitors of Ca^{2+}-ATPase (50 μM quercetin or 5 μM rotenone) inhibited mainly the $^{45}Ca^{2+}$ efflux into calcium-rich medium and had only a slight effect on $^{45}Ca^{2+}$ efflux into calcium-free medium (Table I).

The effect of $1,25(OH)_2D_3$ on the membrane phospholipid composition was studied. The phospholipids of the exterior membranes were analysed in control cells and in cells treated with $1,25(OH)_2D_3$ for 4 hours. As shown in Table II, $1,25(OH)_2D_3$ caused an increase in phosphatidylcholine (PC) and a decrease in phosphatidylethanolamine (PE) content. The ratio of PC/PE increased from 1.2 to 1.5 in the $1,25(OH)_2D_3$-treated cells.

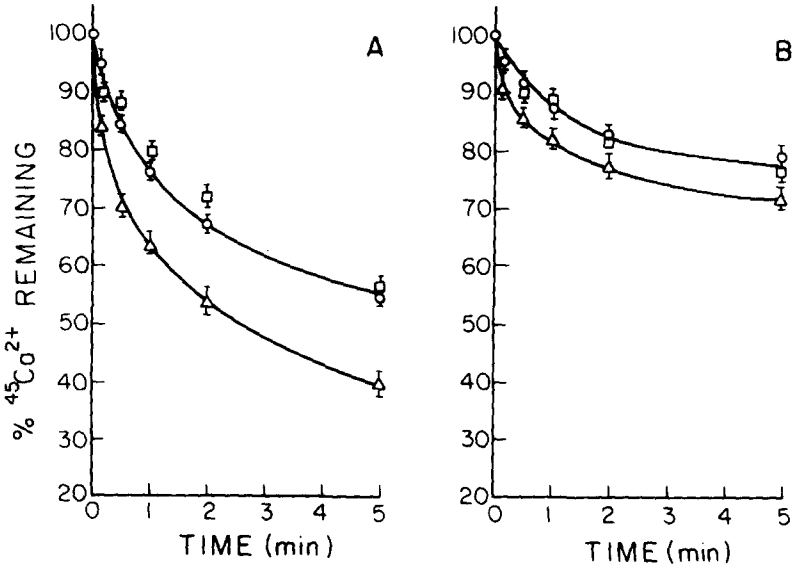

Fig. 3. The effect of vitamin D metabolits on $^{45}Ca^{2+}$ efflux from HL-60 cells to calcium-free medium (A) and calcium-rich medium (B). The cells were treated with 10^{-7} M of the vitamin D metabolites for 4 h prior to the Ca^{2+} efflux determination. Values are means ± S.E. of three experiments. Calcium-free medium contains 2 mM EGTA. Calcium-rich medium contains 2 mM Ca^{2+}. Symbols: ○, control; △, $1,25(OH)_2D_3$; □, $25(OH)D_3$.

TABLE I

THE EFFECT OF Ca^{2+}-ATPase INHIBITORS ON THE $^{45}Ca^{2+}$ EFFLUX FROM HL-60 CELLS

The results are means ± S.E. from three experiments, each in duplicate.

	% $^{45}Ca^{2+}$ remaining		
	Control	+ rotenone (5 μM)	+ quercetin (50 μM)
Ca-free medium	63 ± 2	70 ± 4	74 ± 2
Ca-rich medium	82 ± 4	97 ± 4	95 ± 3

TABLE II

THE EFFECT OF $1,25(OH)_2D_3$ ON THE MEMBRANE PHOSPHOLIPID COMPOSITION OF THE HL-60 CELLS

The cells were treated with 10^{-7} M $1,25(OH)_2D_3$ for 4 h. The results are the means ± S.E. of five experiments.

Compound	Phospholipid distribution (mole %)	
	Control	+ $1,25(OH)_2D_3$
Phosphatidylinositol	7.7 ± 1.55	7.9 ± 0.72
Phosphatidylserine	10.5 ± 1.46	10.5 ± 1.84
Sphingomyelin	10.4 ± 1.33	9.3 ± 1.07
Phosphatidylcholine	38.6 ± 0.75	42.0 ± 0.91 [a]
Phosphatidylethanolamine	31.4 ± 1.24	27.1 ± 1.72 [a]

[a] Significance of difference between the treated cells and the control $P < 0.01$.

Discussion

The results obtained in the present study suggest a direct effect of $1,25(OH)_2D_3$ on the HL-60 cell membrane, $1,25(OH)_2D_3$ caused an increase in $^{45}Ca^{2+}$ efflux from HL-60 cells. The efflux into Ca^{2+}-rich medium may represent the active calcium transport, as it was inhibited by Ca^{2+}-ATPase inhibitors, while the efflux into calcium-free medium represents mostly the passive transport. Since the effect of $1,25(OH)_2D_3$ was more pronounced in the Ca^{2+} efflux into calcium-free medium, it is suggested that the main effect of $1,25(OH)_2D_3$ is on the passive calcium efflux. The increase in the $^{45}Ca^{2+}$ efflux caused by $1,25(OH)_2D_3$ was found to be dose dependent, with a maximal effect at 10^{-7} M. The same concentration of $1,25(OH)_2D_3$ was found previously [20,21] to inhibit the proliferation of the HL-60 cells and to induce their differentiation into monocytes with a maximal effect.

The effect of $1,25(OH)_2D_3$ on $^{45}Ca^{2+}$ efflux is apparently specific since it did not occur in its closely related structural analog, $25(OH)D_3$. The specificity of the effect of $1,25(OH)_2D_3$ on Ca^{2+} transport was also shown by Giuliani and Boland [22,23], who demonstrated a direct effect of $1,25(OH)_2D_3$, increasing the rate constant of radiolabelled calcium efflux from soleus muscle.

The $1,25(OH)_2D_3$ treatment which was found to increase the Ca^{2+} efflux from the HL-60 cells also caused alterations in the phospholipid composition of the outer membranes. The PC content in the membrane increased and the PE content decreased. The increased PC/PE ratio in the $1,25(OH)_2D_3$-treated HL-60 cells membranes in the present study is in agreement with previous results. Rasmussen et al. [9,24], using brush-border membrane vesicles prepared from chick duodenal mucosal cells, showed an increase in the PC/PE ratio within 1–1.5 h, which was maximal at 2–3 h. Similar to our results, the increase in the rate of Ca^{2+} transport was seen within 1.5–2 h and was maximal at 4–6 h. These data demonstrate that the changes in membrane lipid structure either slightly precede or occur simultaneously with the change in Ca^{2+} transport rate, and are therefore consistent with the hypothesis that changes in membrane lipid structure are responsible for the change in Ca^{2+} transport rate.

Changes in phospholipid composition in membranes brought about by $1,25(OH)_2D_3$ were also shown by Tsutsumi et al. [25]. These investigators studied the effect of $1,25(OH)_2D_3$ on the phospholipid composition of rat renal brush border membranes. The PC content in the renal brush border was significantly lower in rats deprived of vitamin D for 5–6 weeks compared to rats receiving a vitamin D supplement for 2 weeks. Moreover, a single physiological dose of $1,25(OH)_2D_3$ (30 pmol) for 16 h to vitamin D-depleted rat renal brush-border membranes caused an increase in their PC content. Matsumoto et al. [10] studied the effect of $1,25(OH)_2D_3$ on phospholipid metabolism in a clonal osteoblast-like rat osteogenic sarcoma cell line. They showed that treatment of these cells with $1,25(OH)_2D_3$ for 48 h caused significant changes in phospholipid

metabolism. $1,25(OH)_2D_3$ markedly enhanced the incorporation of [^{14}C]serine into phosphatidylserine (PS) and suppressed the incorporation of [^{3}H]ethanolamine into PE. Although the PC content in the membrane of these cells was not significantly altered by $1,25(OH)_2D_3$, the PC/PE ratio was increased. Elgavish et al. [26] studied a model system in which vitamin D-3 was incorporated into phosphatidylcholine liposomes which were then incubated with isolated renal brush-border membrane vesicles. The incubation resulted in alterations of the phospholipid composition, the fluidity and the transport properties of the membrane. The findings provided evidence consistent with the hypothesis that vitamin D-3 modifies membrane structure and function by 'liponomic regulation'.

It may be concluded that the short-term effect of $1,25(OH)_2D_3$ caused an increase in the PC/PE ratio in the membranes. Such changes are known to increase the fluidity of the membranes [26,27] and may thus be responsible for the increased Ca^{2+} efflux through the cells. The fact that changes in the phospholipid composition in the membranes by $1,25(OH)_2D_3$ were observed not only in chick duodenal mucosal membraes [7,10] but also in renal brush-border membranes [24] and in human leukemic cells, as shown in the present study, indicates a possible general role of $1,25(OH)_2D_3$ in Ca^{2+} transport in various types of cells, via a direct effect on phospholipid membrane composition.

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