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Rapid actions of 1α,25-dihydroxyvitamin D_3 on Ca^{2+} and phospholipids in isolated rat liver nuclei

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The effects of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) on Ca^{2+} levels and phospholipid metabolism were studied in isolated nuclei prepared from rat liver. Nuclear Ca^{2+} concentration was estimated with the fluorescent indicator Fura 2. In agreement with previous reports, ATP (1 mM) produced a rapid increase in nuclear Ca^{2+} from 188±25 to 593±121 nM. Exposure to 1α,25-$(OH)_2D_3$ (20 nM) also produced a rapid increase in nuclear Ca^{2+} to 402±71 nM. The 1β epimer of 1α,25-$(OH)_2D_3$ had no effect. Nuclear phosphatidylinositol was labeled by incubation with [γ-^{32}P]ATP for 3 h. 1α,25-$(OH)_2D_3$ produced a two-fold increase in [^{32}P]lysophosphatidylinositol (LPI) within 5 min from 44±11 to 87±19 cpm/2.5 × 10^7 nuclei. 1β,25-$(OH)_2D_3$ had no effect on [^{32}P]LPI production. Exposure of nuclei to exogenous LPI (15 μM) produced an instantaneous increase in nuclear Ca^{2+} to 372±81 nM, comparable to ATP and 1α,25-$(OH)_2D_3$. The rapid effects of 1α,25-$(OH)_2D_3$ on phospholipid metabolism and Ca^{2+} in isolated nuclei suggest that the steroid may exert effects distinct from the well-characterized receptor-mediated changes in gene expression.

1α,25-$(OH)_2D_3$; Ca^{2+}, nuclear; Phosphatidylinositol, nuclear

1. INTRODUCTION

Ca^{2+} plays an important role in the regulation of nuclear function. Intracellular Ca^{2+} may serve as a signal to regulate gene expression and the growth of transformed cells [1,2]. Increases in intracellular Ca^{2+} have been shown to rapidly alter the expression of the *fos* and *jun* proto-oncogenes [3,4] and increments in cell and nuclear Ca^{2+} precede DNA fragmentation [5–7]. The ion also stimulates poly(ADP-ribosylation) of nuclear proteins [6]. Poly(ADP-ribosylated) histones appear to be intermediates in nuclear processes involving DNA strand breaks [8].

The steroid hormone, 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), has traditionally been thought to simply play a role in the regulation of plasma Ca^{2+}. However, in addition to its effects on extracellular Ca^{2+}, the seco-steroid rapidly and specifically increases intracellular Ca^{2+} in hepatocytes [9–12] and a variety of other cell types [13–16]. The hormone has also been shown to regulate a variety of gene products [17–22]. The physiologic consequences of these nuclear effects of the hormone include alterations in cell proliferation and differentiation [17]. Since both intracellular Ca^{2+} and 1α,25-$(OH)_2D_3$ appear to be involved in nuclear function and since Ca^{2+} has been reported to modulate the effect of 1α,25-$(OH)_2D_3$ on calbindin gene expression [23,24], we tested the hypothesis that the seco-steroid alters nuclear Ca^{2+} content. The results demonstrate that the hormone rapidly increased Ca^{2+} within the nucleus and altered nuclear phospholipid metabolism. These rapid nuclear actions of 1α,25-$(OH)_2D_3$ may serve to modulate the genomic effects of the hormone.

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2. MATERIALS AND METHODS

Male Sprague–Dawley rats, 250–300 g, were sacrificed under ketamine anesthesia. The livers were removed, homogenized, and fractionated by differential centrifugation by the method of Nicotera [7] to isolate the nuclei. The purified nuclei, confirmed by phase contrast microscopy, were resuspended in medium containing: 125 mM KCl, 2 mM K_2HPO_4, 25 mM Hepes, 4 mM $MgCl_2$, and 2 mM EGTA, pH 7.0. Ca^{2+} was added to an estimated free concentration of 200 nM to approximate cytosolic Ca^{2+} concentrations. Nuclei, 1 × 10^7/ml, were loaded with 1 μM Fura 2/AM (Molecular Probes, Eugene, OR) for 1 h at 4°C. Nuclei were washed, resuspended, and equilibrated at 37°C under an atmosphere of 95% O_2:5% CO_2 for 15 min.

Nuclear Ca^{2+} was estimated from the fluorescence of Fura 2 [7] determined with a Perkin-Elmer 650-10s scanning fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). Fluorescence intensity was monitored at an emission wavelength of 510 nm and with the excitation wavelength alternating between 340 and 380 nm. Nuclear Ca^{2+} content was calculated by the equation:

$$Ca^{2+} = K_d(R - R_{min})/(R_{max} - R)[S_{f2}/S_{b2}]$$

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where fluorescence (R) is the ratio of fluorescence at 340/380, maximal fluorescence (R_{max}) is the ratio at 340/380 of nuclei lysed by the addition of Triton X-100, and minimal fluorescence (R_{min}) is the ratio at 340/380 of nuclei incubated in the presence of EGTA to chelate Ca^{2+}. The K_d was calculated to be 182 nM and the S_{f2}/S_{b2} constant 2.82 in our system [10]. 1α,25-$(OH)_2D_3$ (courtesy of Dr M. Uskovic, Hoffmann-La Roche, Inc., Nutley, NJ), 1β,25-dihydroxyvitamin D_3 (1β,25-$(OH)_2D_3$), lysophosphatidylinositol (LPI) (Avanti, Birmingham, AL), and ATP (Boehringer Mannheim, Indianapolis, IN) were added in a volume of 0.01 ml to 1 ml of nuclei at 37°C.

Phospholipids in rat liver nuclei were labeled by incubation with [γ-^{32}P]ATP (spec. act. 2 mCi/ml, Dupont, Boston, MA) at a concentration of 0.01 mCi/10^7 nuclei for 3 h before exposure to alcohol, 1α,25-$(OH)_2D_3$ (20 nM), or 1β,25-$(OH)_2D_3$ (20 nM) for 5 min. Nuclei were centrifuged at 250 × g for 1 min and the nuclear pellet dissolved in 3 ml of CH_3Cl:CH_3OH:HCl (100:50:1). 0.2 M KCl solution (1 ml) was added to each sample, and phases separated by centrifugation at 250 × g for 1 min. The aqueous phase was removed and the lower lipid phase was re-extracted with 1 ml of 0.2 M KCl:CH_3OH (1:1). The lipid fraction was dried, dissolved in 50 μl $CHCl_3$, and applied to preactivated (110°C for 30 min) 10 × 10 cm silica gel 60 HPTLC plates (Merck, Darmstadt, FRG). The phospholipids were separated by development in the first dimension with $CHCl_3$:CH_3OH:NH_4OH (60:30:7) and at right angles with $CH_3(CH_2)_3OH$:CH_3CO_2H:H_2O (6:1:1) in the second phase. Phosphatidylinositol (PI) (Sigma, St. Louis, MO) and LPI standards were cochromatographed for correct identification of individual phospholipids [12]. Phospholipids were detected on individual plates by exposure to 6-p-toluidino-2-naphthalene sulfonic acid (TNS) (Eastman Kodak, Rochester, NY) [25] and by autoradiography on Kodak Diagnostic film X-OMAT-AR. A fine mist of the TNS solution (1 mM TNS in 50 mM Tris-HCl, pH 7.4) was sprayed on the plates, identifying the phospholipids as bright spots on a dark background. Radioactivity in the individual phospholipids was quantified by scraping the identified spot and counting in 3 ml Econofluor (New England Nuclear/Dupont, Boston, MA) in a Beckman LS 1701 Liquid Scintillation Counter.

3. RESULTS AND DISCUSSION

In agreement with previous reports [7], ATP (1 mM) rapidly increased the Ca^{2+} concentration in isolated nuclei as measured by an increase in the fluorescence of the Ca^{2+}-sensitive probe Fura 2 (fig.1). 1α,25-$(OH)_2D_3$ produced a similar effect in isolated nuclei, although lesser in magnitude (fig.1). 1β,25-$(OH)_2D_3$, an epimer of 1α,25-$(OH)_2D_3$ which does not affect Ca^{2+} metabolism, did not alter nuclear fluorescence (fig.1). LPI, a lysolipid which mimics the 1α,25-$(OH)_2D_3$ effects on cytosolic Ca^{2+} in isolated hepatocytes [12], also increased nuclear Ca^{2+} (table 1). After 5 min of exposure to agonists, the nuclear free Ca^{2+} concentration was increased by ATP, 1α,25-$(OH)_2D_3$, and LPI, while 1β,25-$(OH)_2D_3$ remained ineffective (table 1).

Cytosolic Ca^{2+} is regulated in part by changes in PI metabolism. To determine whether phospholipid metabolism in the nucleus was altered by agonists, we examined PI metabolism in the isolated liver nuclei. Nuclear PI was labeled with ^{32}P by incubation with [γ-^{32}P]ATP. Exposure of nuclei to 1α,25-$(OH)_2D_3$ produced a rapid increase in [^{32}P]LPI, suggesting the steroid may alter phospholipase A_2 in this nuclear system (table 2). Concomitant with the increase in nuclear free Ca^{2+}, 1α,25-$(OH)_2D_3$ enhanced the production of [^{32}P]LPI in the nuclear membrane by 98%, while 1β,25-$(OH)_2D_3$ was without effect (table 2).

Previous studies from our laboratory have demonstrated that 1α,25-$(OH)_2D_3$ rapidly increases

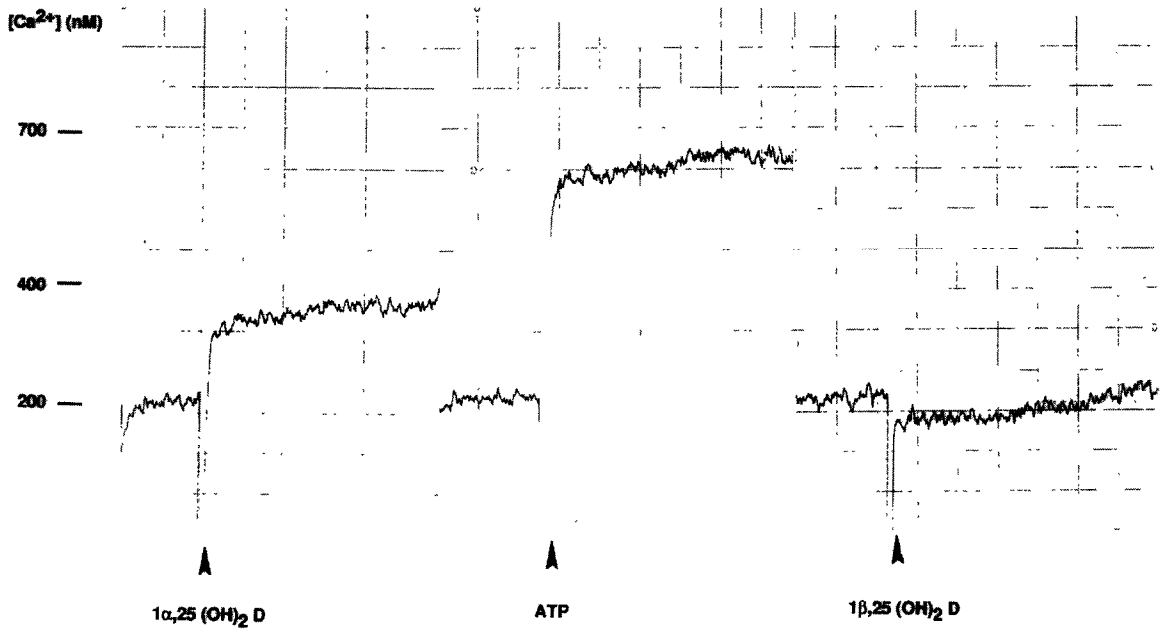

Fig.1. Fura 2 fluorescence in isolated nuclei. To continuously monitor nuclear Ca^{2+}, the changes in Fura 2 fluorescence were determined at 340 nm excitation and the Ca^{2+} content estimated from changes in fluorescence. Representative tracings are shown. For convenience, the fluorescent intensity is expressed as apparent Ca^{2+} content. The rapid alterations in fluorescence shown here at excitation 340 nm correspond to calculated Ca^{2+} concentrations.

Table 1

Increase in the apparent free Ca^{2+} concentration of isolated rat liver nuclei after exposure to ATP, $1\alpha,25\text{-}(OH)_2D_3$, $1\beta,25\text{-}(OH)_2D_3$, or LPI

	Ca^{2+} (nM)	Increase (%)
Control ($n = 9$)	188 ± 25	-
ATP (1 mM), ($n = 7$)	593 ± 121	215*
$1\alpha,25\text{-}(OH)_2D_3$ (20 nM), ($n = 9$)	402 ± 71	114**
$1\beta,25\text{-}(OH)_2D_3$ (20 nM), ($n = 5$)	210 ± 68	12
LPI (15 μM), ($n = 7$)	372 ± 81	98**

The free Ca^{2+} concentration was measured by Fura 2 fluorescence after 5 min exposure to agonists. Values represent the mean ± SD. Numbers in parentheses (n) indicate the number of observations. Probability of difference was determined by Duncan's test for multiple comparisons

* Significantly greater than all other groups ($P<0.05$)
**Significantly greater than control and $1\beta,25\text{-}(OH)_2D_3$ ($P<0.05$)

hepatocyte cytosolic Ca^{2+}, and this action appears to be linked to changes in phospholipid metabolism, specifically the generation of LPI [9–12]. In the present studies, $1\alpha,25\text{-}(OH)_2D_3$ was shown to exert rapid, stereospecific effects on Ca^{2+} and phospholipid metabolism in a cell-free system, isolated nuclei. Since $1\alpha,25\text{-}(OH)_2D_3$ produced an increase in LPI (table 2) over the same time period as an increase in nuclear Ca^{2+} (table 1), and exposure of isolated nuclei to LPI also increased Ca^{2+} (table 1), the data suggest that this lysolipid may serve as a signal for these rapid nuclear actions of $1\alpha,25\text{-}(OH)_2D_3$.

The actions of $1\alpha,25\text{-}(OH)_2D_3$ were stereospecific in that the epimer $1\beta,25\text{-}(OH)_2D_3$ failed to produce an increase in nuclear Ca^{2+} (table 1) or LPI (table 2). Similarly, the 1β epimer does not alter cytosolic Ca^{2+} or LPI in intact hepatocytes [12]. This epimer is unable to increase plasma Ca^{2+} or enhance intestinal Ca^{2+} transport [26]. The lack of effect of $1\beta,25\text{-}(OH)_2D_3$ on intestinal Ca^{2+} transport has been attributed to its inability to bind to, or to displace $1\alpha,25\text{-}(OH)_2D_3$ from, the cytosolic receptor thought to mediate this action [26].

Although ATP, $1\alpha,25\text{-}(OH)_2D_3$, and LPI all increased nuclear Ca^{2+}, the effects of the hormone and LPI were apparent in the absence of ATP. Therefore, exogenous nucleotide is not necessary for $1\alpha,25\text{-}(OH)_2D_3$- and LPI-induced increments in nuclear Ca^{2+}. Previous studies have suggested that nuclear Ca^{2+} accumulation is the result of active transport of Ca^{2+}, based upon ATP dependency and modulation by inhibitors of calmodulin [7]. Neither $1\alpha,25\text{-}(OH)_2D_3$ nor LPI would directly provide a suitable energy source for such active transport. If increased nuclear Ca^{2+} truly reflects accumulation against an electrochemical gradient, the effects of $1\alpha,25\text{-}(OH)_2D_3$ and LPI may be the result of lysolipid-induced alterations in the nuclear membrane properties to other ions, such that the Ca^{2+} gradient is altered and nuclear Ca^{2+} content is increased.

Table 2

Increase in [^{32}P]lysophosphatidylinositol (LPI) in isolated rat liver nuclei after exposure to $1\alpha,25\text{-}(OH)_2D_3$

	[^{32}P]LPI (cpm/2.5 × 10^7 nuclei)	Increase (%)
Control ($n = 6$)	44 ± 11	-
$1\alpha,25\text{-}(OH)_2D_3$ (20 nM), ($n = 5$)	87 ± 19*	98*
$1\beta,25\text{-}(OH)_2D_3$ (20 nM), ($n = 4$)	47 ± 19	7

Values represent the mean ± SD. Numbers in parentheses (n) indicate the number of observations in each group. Probability of difference was determined by Duncan's test for multiple comparisons

*Significantly greater than other groups ($P<0.05$)

Exogenous nucleotide is necessary to generate new phospholipid synthesis which we can detect by autoradiography (table 2). It appears that $1\alpha,25\text{-}(OH)_2D_3$ can induce the deacylation of pre-existing nuclear phospholipids, perhaps by activating phospholipase A present in the nuclear matrix [27], to generate the LPI signal and subsequent Ca^{2+} increments. $1\alpha,25\text{-}(OH)_2D_3$ has previously been shown to increase phospholipase A activity in intact hepatocytes [11,12]. Like Ca^{2+}, the phospholipid environment has been postulated to be important in the regulation of nuclear functon. The inositol phospholipids in particular have been shown to modulate *ras* gene activity during mitogenic stimulation [28–30] and to stimulate the initiation of DNA synthesis [31]. Recent studies indicate that phospholipid phosphorylation takes place in isolated rat liver nuclei [32]. The data suggested that the nucleus, and especially the nuclear periphery, is a cell compartment in which polyphosphoinositide synthesis occurs; this might be related to the progression of PI metabolism-dependent signals to the genetic apparatus [32]. We have not only confirmed that phospholipid metabolism takes place in the nucleus, but have demonstrated that it can be hormonally modulated (table 2).

In conclusion, this study demonstrates rapid, stereospecific effects of $1\alpha,25\text{-}(OH)_2D_3$ on the free Ca^{2+} of isolated rat liver nuclei. The hormone also rapidly and stereospecifically alters the deacylation of PI to LPI, which in turn can increase nuclear free Ca^{2+}. These $1\alpha,25\text{-}(OH)_2D_3$ effects on nuclear Ca^{2+} and phospholipids may modulate the hormone's actions on cell growth and oncogene expression.

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