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1,25-Dihydroxycholecalciferol Modulates ^{3}H-Thymidine Incorporation in FRTL5 Cells

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Abstract 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) possesses proliferation and differentiation modulating effects in many cell types in vitro. We studied the effect of $1,25(OH)_2D_3$ on ^{3}H-thymidine incorporation in FRTL5 cells, a cultured rat thyroid follicular cell line. $1,25(OH)_2D_3$ alone at 10^{-11} and 10^{-9} M exerted no effect on ^{3}H-thymidine incorporation. However, at 10^{-7} M, $1,25(OH)_2D_3$ slightly enhanced ^{3}H-thymidine incorporation. In the presence of 5% calf serum, $1,25(OH)_2D_3$ increased ^{3}H-thymidine incorporation induced by calf serum in a dose-dependent manner. $1,25(OH)_2D_3$ also enhanced ^{3}H-thymidine incorporation induced by PMA, an extrinsic stimulator of protein kinase C, without directly affecting PMA-induced protein kinase C translocation. In contrast to the stimulatory effects of $1,25(OH)_2D_3$ on the calf serum and PMA-induced ^{3}H-thymidine incorporation, $1,25(OH)_2D_3$ inhibited the increase in ^{3}H-thymidine incorporation induced by TSH in a dose-dependent manner. This effect of $1,25(OH)_2D_3$ on TSH-induced ^{3}H-thymidine incorporation may be, in part, due to post-cAMP pathways since $1,25(OH)_2D_3$ also inhibited the increase in ^{3}H-thymidine incorporation induced by Bu_2cAMP without affecting the TSH-induced increase in cAMP. The stimulatory effect of insulin on ^{3}H-thymidine incorporation, a cAMP-independent process, was also inhibited by $1,25(OH)_2D_3$. We conclude that $1,25(OH)_2D_3$ affects ^{3}H-thymidine incorporation in FRTL5 cells raising the possibility of a physiologic role for $1,25(OH)_2D_3$ in the growth and function of thyroid follicular cells.

Key words: thyroid follicular cells, cell proliferation, cAMP, protein kinase C

1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) exerts proliferation and differentiation modulating effects in a variety of cell types [Abe et al., 1981; Koeffler et al., 1985; Tsoukas et al., 1984; Frampton et al., 1987; Hosomi et al., 1983; Carthy et al., 1989] besides its well-known action on mineral homeostasis. These effects occur in cells not previously thought to be $1,25(OH)_2D_3$-dependent [Holick and Adams, 1990]. Binding of $1,25(OH)_2D_3$ to thyroid follicular and parafollicular chief cells has been previously described [Stumpf and O'Brien, 1987]. Moreover, the in vivo administration of $1,25(OH)_2D_3$ to experimental animals results in an enhanced response of TSH to TRH administration, suggesting interaction between $1,25(OH)_2D_3$ and the hypothalamic-pituitary-thyroid axis [Smith et al., 1989].

FRTL5 cells, a cultured rat thyroid follicular cell line, have been widely used to study the regulation of thyroid cell growth. Intracellular signal transduction mediating regulation of thyroid cell growth largely involves a cAMP-dependent protein kinase pathway mediated through the TSH receptor [Tramontrano et al., 1984], a cAMP-independent pathway mediated through insulin or insulin-like growth factor-1 (IGF-1) [Tramontrano et al., 1987], and a recently described protein kinase C pathway [Lombardi et al., 1988]. Moreover, arachidonic acid metabolism is also thought to be involved in the regulation of thyroid cell growth and function [Marocci et al., 1987]. All these signal transduction pathways interact in an as yet not clearly defined manner to exert regulatory effects on thyroid follicular cells.

In the present study, we used FRTL5 cells to investigate the effect of $1,25(OH)_2D_3$ on ^{3}H-thymidine incorporation as mediated by various signal transduction pathways.

MATERIALS AND METHODS

Materials

$1,25(OH)_2D_3$ was a gift from Dr. M.R. Uskokovic, Hoffman La Roche, Nutley, NJ. The

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$1,25(OH)_2D_3$ was dissolved in absolute ethanol to a final concentration of 10^{-3} M and stored at −20°C until used. Coon's modified Ham's F12 medium, bovine TSH, bovine insulin, human transferrin, phorbol-13-acetate-12-myristate (PMA), and Bu_2cAMP were obtained from Sigma Chemical Co. (St. Louis, MO); calf serum from Gibco (Grand Island, NY); [methy-^{3}H]thymidine (6.5 mCi/mmol) from New England Nuclear Corp. (Boston, MA); cAMP RIA kit from Incstar (Stillwater, MN); and protein kinase C assay system from Gibco.

Culture of FRTL5 Cells

FRTL5 cells were routinely cultured in Coon's modified Ham's F12 medium supplemented with 5% calf serum and 1 mU/ml TSH, 1 μg/ml insulin, and 5 μg/ml transferrin (3H) under 5% CO_2 and 95% air at 37°C. Medium was changed every 2–3 days and cells were subcultured when confluent, as previously described [Tramontrano and Ingbar, 1986].

^{3}H-Thymidine Incorporation

Cells were seeded in 24-well plates in triplicate and grown in Coon's modified Ham's F12 medium supplemented with 5% calf serum, 1 mU/ml TSH, 1 μg/ml insulin, and 5 μg/ml transferrin. After 3 days, medium was changed to Coon's modified Ham's F12 medium without 3H. Five days later, cells were washed with medium containing no calf serum or 3H. Five hundred microliters of medium containing 0.1% BSA and test agents were then added. In the experiments with $1,25(OH)_2D_3$, the concentrations of ethanol were less than 0.001%. After 40–48 hours, medium was removed and medium containing 5 μCi/ml ^{3}H-thymidine was added. Cells were incubated at 37°C for 3 h and then washed with ice-cold PBS and 10% TCA, respectively. Wells were dried and 500 μl 2% SDS was added to each well. After 30 min, radioactivity in the supernatant was counted in a scintillation spectrometer.

Measurement of cAMP

Cells were seeded and treated before adding the test agents as above and then washed with KRB containing 0.1% glucose, 0.1% BSA, and 1 mM isobutylmethylxanthine. Test agents were added to triplicate wells in 250 μl of the same buffer and incubated for 30 min at 37°C. Medium was aspirated and saved. Then 500 μl of absolute ethanol was added to each well and kept at −20°C overnight. Supernatant was dried and reconstituted with the previously saved medium. cAMP was assayed by RIA.

Assay of Protein Kinase C Activity

Cells were seeded in 100-mm tissue culture dishes and treated before adding the test agents as described above. Agents to be tested were added in Coon's modified Ham's F12 medium and cells were incubated for 15 min. Cells were then washed with ice-cold PBS and scraped into 500 μl of extraction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 25 μg/ml aprotinin, and 25 μg/ml leupeptin). Cells were sonicated for 10 sec and the cytosolic fraction was separated by centrifugation at 100,000*g* for 30 min. The pellet was dissolved in extraction buffer with Triton X-100 for 30 min, centrifuged at 100,000*g* for 30 min, and the supernatant was used as the membrane fraction. Partial purification of the cytosolic and membrane fractions was performed by passing through DEAE Bio-Gel A in chromatographic columns and eluted with 3 ml buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.2 M NaCl) per column. Protein content in the eluate was measured by the method of [Lowry et al., 1951]. Protein kinase C activities were then assayed by measuring phosphorylation of a myelin basic peptide [Yasuda et al., 1990] in the presence and absence of a protein kinase C pseudosubstrate inhibitor peptide [House and Kemp, 1987] at 30°C for 10 min. The final protein kinase C activity was calculated by subtracting the value with inhibitor from that without inhibitor and dividing by the protein content in the eluate. All assays were performed in duplicate and data expressed as the percentage of the sum of cytosolic and membrane protein kinase C activities.

Statistical Analyses

Values represent the mean ± SE and the probability of difference between pairs of data was determined by the Student's *t* test. Multiple comparison was done by analysis of variance (ANOVA) followed by the Duncan's multiple range test where appropriate.

RESULTS

$1,25(OH)_2D_3$ at 10^{-11} and 10^{-9} M, in the absence of calf serum, had no effect on ^{3}H-thymidine incorporation. $1,25(OH)_2D_3$ (10^{-7} M) slightly increased ^{3}H-thymidine incorporation (268 ± 57 cpm/well vs. 595 ± 47, $P < 0.01$). Serum alone significantly increased thymidine incorporation. In presence of calf serum, $1,25(OH)_2D_3$ increased ^{3}H-thymidine incorporation in a dose-dependent manner ($P < 0.01$) (Fig. 1).

Phorbol-12-myristate-13-acetate (PMA, 300 nM), an extrinsic activator of PKC, increased ^{3}H-thymidine incorporation in FRTL5 cells as previously described [Lombardi et al., 1988] (268 ± 57 cpm/well vs. 794 ± 68, $P < 0.01$). The PMA effect was enhanced by 10^{-11} and 10^{-9} $1,25(OH)_2D_3$ ($P < 0.01$) (Fig. 2). PMA increased the percent of PKC activity associated with the membrane fraction with a concomitant decrease in that associated with the cytosolic fraction, evidence of PKC activation. However, $1,25(OH)_2D_3$ did not enhance this effect of PMA (Table I).

TSH, 1 mU/ml, enhanced ^{3}H-thymidine incorporation into FRTL5 cells (268 ± 57 cpm/well vs. 31,253 ± 1,217, $P < 0.01$). $1,25(OH)_2D_3$ inhibited the TSH stimulated ^{3}H-thymidine incorporation in a dose-dependent manner ($P < 0.05$) (Fig. 3). Since the effect of TSH on proliferation and differentiated functions in FRTL5 cells is known to be, in part, due to cAMP-mediated pathways, we studied the effect of $1,25(OH)_2D_3$ on TSH-induced increase in cAMP and the interaction between $1,25(OH)_2D_3$ and Bu_2cAMP which directly activates cAMP-dependent processes. TSH, 1 mU/ml, increased cAMP production as previously described. $1,25(OH)_2D_3$, 10^{-11} to 10^{-7} M, had no effect on cAMP and did not inhibit the increase in cAMP induced by TSH (Table II). Bu_2cAMP, 100 μM, increased ^{3}H-thymidine incorporation (268 ± 57 cpm/well vs. 8,343 ± 762, $P < 0.01$). 10^{-7} M $1,25(OH)_2D_3$ inhibited Bu_2cAMP-induced ^{3}H-thymidine incorporation ($P < 0.05$) (Fig. 4).

Since insulin increases proliferation in FRTL5 cells by a cAMP-independent mechanism, we evaluated the effect of $1,25(OH)_2D_3$ on insulin-induced ^{3}H-thymidine incorporation. Insulin, 10 μg/ml, increased ^{3}H-thymidine incorporation (295 ± 54 cpm/well vs. 35,380 ± 3,426, $P < 0.01$). The increase in ^{3}H-thymidine incorporation induced by insulin was inhibited by

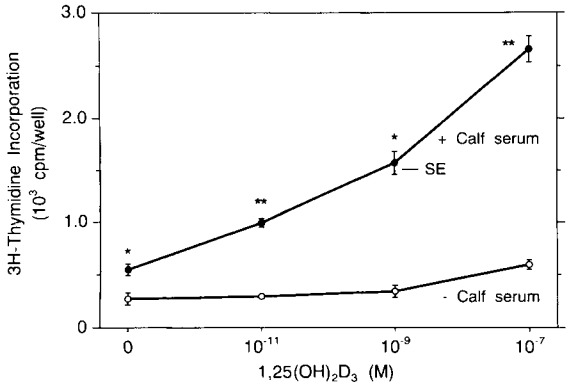

Fig. 1. Effect of $1,25(OH)_2D_3$ on ^{3}H-thymidine incorporation by FRTL5 cells in the presence (+) or absence (−) of 5% calf serum. Values represent the mean ± SE of three observations at each concentration. *$P < 0.01$, **$P < 0.001$ vs. no calf serum at the same $1,25(OH)_2D_3$ concentration.

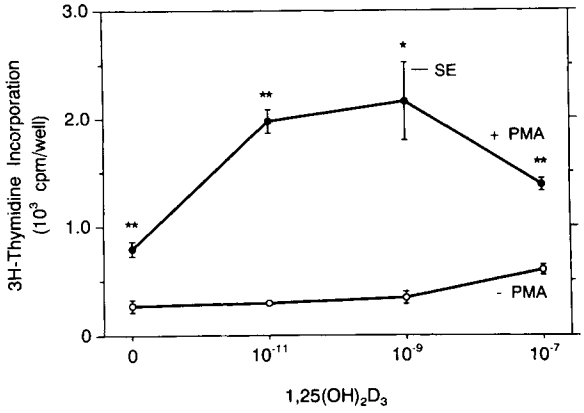

Fig. 2. Effect of $1,25(OH)_2D_3$ on ^{3}H-thymidine incorporation by FRTL5 cells in the presence (+) or absence (−) of PMA. Values represent the mean ± SE of three observations at each concentration. *$P < 0.05$, **$P < 0.01$ vs. no PMA at the same $1,25(OH)_2D_3$ concentration.

TABLE I. Effect of PMA (100 nM), $1,25(OH)_2D_3$ (10^{-9} M), and PMA Plus $1,25(OH)_2D_3$ on the Percent Protein Kinase C Activity Associated With Cytosolic and Membrane Fractions[a]

	Cytosol (% total)	Membrane (% total)
Control	72.6 ± 3.2	27.5 ± 3.2
PMA	21.1 ± 2.6*	78.9 ± 2.6*
$1,25(OH)_2D_3$	72.2 ± 0.9	27.8 ± 0.9
PMA + $1,25(OH)_2D_3$	25.6 ± 0.7*	74.4 ± 0.7*

[a]Values represent the mean ± SE. Similar results were obtained in another separate experiment.
*$P < 0.001$ compared to control.

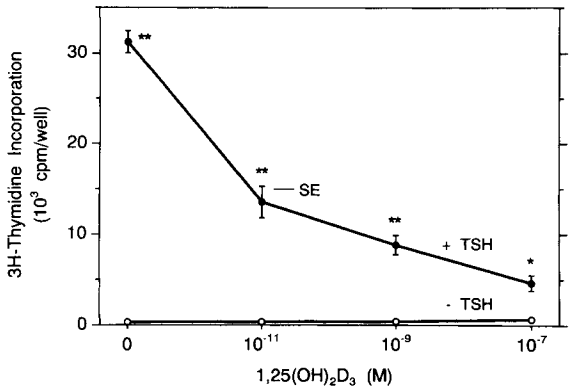

Fig. 3. Effect of $1,25(OH)_2D_3$ on ^{3}H-thymidine incorporation by FRTL5 cells in the presence (+) or absence (−) of TSH. Values represent the mean ± SE of three observations at each concentration. $*P < 0.05$, $**P < 0.001$ vs. no TSH at the same $1,25(OH)_2D_3$ concentration.

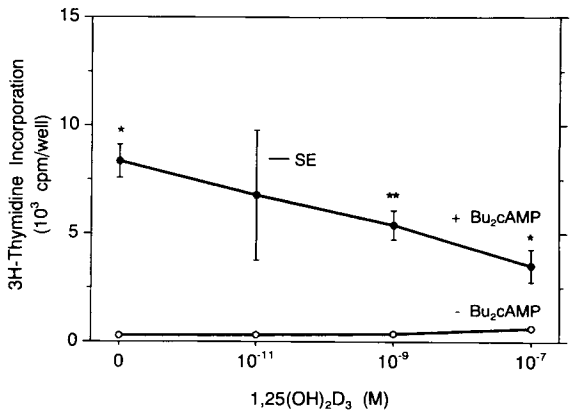

Fig. 4. Effect of $1,25(OH)_2D_3$ on ^{3}H-thymidine incorporation by FRTL5 cells in the presence (+) or absence (−) of Bu_2cAMP. Values represent the mean ± SE of three observations at each concentration. $*P < 0.05$, $**P < 0.01$ vs. no Bu_2cAMP at the same $1,25(OH)_2D_3$ concentration.

TABLE II. Effect of $1,25(OH)_2D_3$ on cAMP Concentration in the Presence and Absence of TSH[a]

	cAMP (nmol/L/well)		
$1,25(OH)_2D_3$ (M)	Without TSH	With TSH	P
0	6.0 ± 1.7	866.7 ± 246.2	<0.05
10^{-11}	8.1 ± 0.7	800.0 ± 173.2	<0.05
10^{-9}	7.0 ± 1.4	965.0 ± 28.9	<0.001
10^{-7}	4.6 ± 1.1	971.7 ± 45.1	<0.01

[a]Values represent the mean ± SE of three observations at each concentration. Similar results were obtained in another separate experiment.

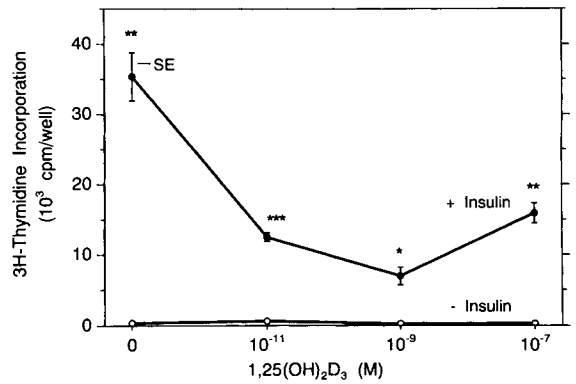

Fig. 5. Effect of $1,25(OH)_2D_3$ on ^{3}H-thymidine incorporation by FRTL5 cells in the presence (+) or absence (−) of insulin. Values represent the mean ± SE of three observations at each concentration. $*P < 0.05$, $**P < 0.01$, $***P <$ vs. no insulin at the same $1,25(OH)_2D_3$ concentration.

10^{-11}, 10^{-9}, and 10^{-7} M $1,25(OH)_2D_3$ ($P < 0.01$) (Fig. 5).

DISCUSSION

The present study demonstrates that $1,25(OH)_2D_3$ modulates ^{3}H-thymidine incorporation in FRTL5 cells. $1,25(OH)_2D_3$ receptors have been reported in these cells [Lamberg-Allardt et al., 1991; Berg et al., 1991]. The physiologic role of $1,25(OH)_2D_3$ in the thyroid is uncertain. However, there is evidence that $1,25(OH)_2D_3$ may affect thyroid function and growth. Administration of $1,25(OH)_2D_3$ to rats enhances the TSH response to TRH [Smith et al., 1989]. In addition, goiters are more prevalent in patients with end-stage renal disease [Hegedus et al., 1985; Kaptein et al., 1988]. Although this goitrogenic response is multifactorial in origin, it is possible that the decrease in $1,25(OH)_2D_3$ synthesis by the kidneys in chronic renal failure may play a role.

$1,25(OH)_2D_3$ alone slightly stimulates ^{3}H-thymidine incorporation in FRTL5 cells, but only at a high concentration. However, $1,25(OH)_2D_3$ modulates the action of other growth promoting agents on ^{3}H-thymidine incorporation. This suggests that $1,25(OH)_2D_3$ may not be essential for thyroid follicular cell growth but may play a modulatory role in the growth of thyroid follicular cells. The mechanism of action of this effect on ^{3}H-thymidine incorporation is uncertain. Nevertheless, it seems to involve more than one signal tranduction pathway. In the case of the protein kinase A pathway, we demonstrated that $1,25(OH)_2D_3$ has an inhibitory effect on the en-

hanced ^{3}H-thymidine incorporation induced by TSH. This effect may be due to post-adenylate cyclase rather than pre-adenylate cyclase effects since $1,25(OH)_2D_3$ inhibited ^{3}H-thymidine incorporation induced by TSH without affecting the TSH-induced increase in cAMP and inhibited the Bu_2cAMP-induced increase in ^{3}H-thymidine incorporation.

In the present study, $1,25(OH)_2D_3$ also enhanced the ^{3}H-thymidine incorporation effect of PMA, an extrinsic stimulator of protein kinase C. PMA has previously been reported to increase c-myc protooncogenes and subsequent cell proliferation in FRTL5 cells [Lombardi et al., 1988]. $1,25(OH)_2D_3$ has been reported to modulate growth in other cell types by interaction with protein kinase C. For example, $1,25(OH)_2D_3$ increases protein kinase C transcription [Obeid et al., 1990] and phorbol ester receptors [Martell et al., 1987] in the HL-60 promonocytic leukemic cell line and the differentiation-inducing effect of $1,25(OH)_2D_3$ on these cells could be blocked by a protein kinase C inhibitor [Martell et al., 1988]. However, although PMA caused an increase in protein kinase C activity associated with the membrane fraction with a concomitant decrease in cytosolic protein kinase C activity, evidence of protein kinase C activation, this action of PMA was not affected by $1,25(OH)_2D_3$. This suggests that $1,25(OH)_2D_3$ may enhance the ^{3}H-thymidine incorporation effect of PMA distal to the activation of protein kinase C.

We also demonstrated that $1,25(OH)_2D_3$ inhibits its ^{3}H-thymidine incorporation induced by high concentration of insulin. This action of insulin in FRTL5 cells is presumably mediated through IGF1 receptors and tyrosine-specific kinase [Tramontrano et al., 1984]. $1,25(OH)_2D_3$ has been reported to decrease IGF-1 receptors in HL-60 leukemia cells [Sukegawa et al., 1987] which may be one of the mechanisms underlying the interaction between $1,25(OH)_2D_3$ and insulin in FRTL5 cells. Other growth factors present in calf serum may account for the findings that $1,25(OH)_2D_3$ enhanced ^{3}H-thymidine uptake induced by calf serum. $1,25(OH)_2D_3$ has been reported to increase epidermal growth factor receptors in a rat bone–derived cell line [Martin Petkovich *et al.*, 1987]. On the other hand, epidermal growth factor has been reported to increase $1,25(OH)_2D_3$ receptors [Brune et al., 1989].

We conclude that $1,25(OH)_2D_3$ affects ^{3}H-thymidine incorporation in FRTL5 cells. This raises the possibility that $1,25(OH)_2D_3$ may have a physiologic role in the growth and function of the thyroid gland.

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