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Ongoing protein synthesis needed for 1,25-$(OH)_2D_3$-mediated rapid increase of cyclic GMP in human skin fibroblasts

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Recently we reported that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) through interaction with its specific receptor rapidly (within 1 min) stimulated intracellular cGMP production in cultured human skin fibroblasts. Here we show that this effect of 100 nM 1,25-$(OH)_2D_3$ is prevented by brief (30 min) inhibition of RNA synthesis (with actinomycin D or α-amanitin) or by brief inhibition of protein synthesis (with cycloheximide or diphtheria toxin). The protein synthesis inhibitors also blocked stimulation of cGMP by other steroids (testosterone or dexamethasone at 100 nM) but did not block cGMP stimulation by sodium nitroprusside. Since the time for the 1,25-$(OH)_2D_3$ receptor to increase cGMP seems too short to require de novo protein synthesis, we conclude that the 1,25-$(OH)_2D_3$ receptor acts together with rapidly turning over protein(s) to stimulate cGMP synthesis.

Calcitriol; Steroid receptor; GMP; Signal transduction; Gene expression

1. INTRODUCTION

It is generally believed that receptor-mediated actions of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) require a 0.5–12 h induction interval, during which selected mRNA and protein concentrations begin to be modulated [1]. Transcriptional and translational effects of steroids may require proteins ancillary to the steroid receptor. Therefore, inhibitors of RNA and protein synthesis could block 1,25-$(OH)_2D_3$ genomic actions either by preventing de novo transcription and translation of mRNA or by preventing production of labile protein(s) participating in the processes of transcription or translation. Such ancillary proteins with rapid turnover have been suggested to participate in regulation of mRNA transcription by several v-ERB-A-related molecules, including the receptors for progesterone [2], glucocorticoid [3], and triiodothyronine [4–6].

1,25-$(OH)_2D_3$, like other steroids, can elicit extremely rapid effects [7]. These rapid actions have generally been considered nongenomic and have sometimes been found to be independent of ongoing RNA and protein synthesis [8,9]. Recently, we found that 1,25-$(OH)_2D_3$, acting through an otherwise typical steroid receptor, elevated intracellular cGMP concentration in human skin fibroblasts within 1–3 min [10]. The short interval preceding the 1,25-$(OH)_2D_3$ receptor-mediated rise in cGMP suggests that this action is not the result of altered mRNA or protein synthesis. We studied the effect of RNA and protein synthesis inhibitors on the 1,25-$(OH)_2D_3$-mediated increase in cGMP.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Normal human skin fibroblasts were grown in Dulbecco's minimal essential medium (Biofluids, Rockville, MD) supplemented with 10% defined fetal bovine serum (HyClone, Logan, UT), 0.2 mM glutamine, 80 mg/l gentamycin, and

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Abbreviation: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3

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0.1 μM insulin as in [10]. Cells were plated at 40000/vial to 24-vial dishes and cultured for 24 h.

2.2. *Perturbation and measurement of cGMP*

Cells in 24-well plates were maintained in serum-free medium for 24 h. Then cells were preincubated for 30 min at 37°C with or without metabolic inhibitors in fresh serum-free media. Actinomycin D (Sigma, St. Louis, MO) at 10 μg/ml or in other experiments 0.1 mg/ml α-amanitin (Sigma) were used to inhibit RNA synthesis. Either 20 μg/ml cycloheximide (Sigma) or 50 nM diphtheria toxin ('nicked', Calbiochem, San Diego, CA) were used to inhibit protein synthesis. After this 30 min preincubation, monolayers were washed with Eagle's no.2 medium without calcium, magnesium, sodium bicarbonate, and containing 25 mM Hepes and 2 mM manganese at pH 7.4. Then cells were incubated for 3–4 min at 37°C with and without agonists [1,25-$(OH)_2D_3$, testosterone, or dexamethasone at 100 nM, or sodium nitroprusside at 1 mM] in the presence of 0.1 mg/ml isobutylmethylxanthine. Reaction was stopped by removal of medium and addition of *n*-propanol. The cGMP content of vials was analyzed by a sensitive radioimmunoassay after acetylation [10]. In each experiment cGMP was measured in wells without drug, in wells with metabolic inhibitor (inhibitor of RNA or protein synthesis), in wells with cGMP agonist (a steroid or sodium nitroprusside), and in wells with metabolic inhibitor plus an agonist. Protein content was also measured (Biorad kit).

2.3. *Measurement of RNA synthesis*

Cells were incubated for 30 min with or without metabolic inhibitor and then incubated for another 1 h in medium containing 5 μCi/ml [^{3}H]uridine (NEN, Dupont, Boston; spec. act. 27.5 Ci/mmol) without metabolic inhibitor. Then the reaction was stopped with removal of medium, and monolayers were washed with ice-cold phosphate-buffered saline. Acid-insoluble materials were precipitated by addition of 500 μl of 5% trichloroacetic acid solution and incubation for 2 h at 4°C. The dried precipitate was then solubilized with 1 N NaCl. Radioactivity was counted in a liquid scintillation counter and data were expressed as % of counts in control wells without inhibitor added (mean ± 1 SE).

2.4. *1,25-$(OH)_2D_3$ binding to soluble extracts from cells*

Immediately before being harvested, cells were incubated in fresh serum-free medium with or without actinomycin D (10 μg/ml) for 30 min. Hormone binding to soluble extracts was measured in triplicate aliquots in two separate experiments, as in [11].

2.5. *Statistics*

Results are shown as mean ± SE. Paired *t*-test was used for data analysis.

3. RESULTS

3.1. *Effects of transcription inhibitors on the rapid cGMP rise caused by 1,25-$(OH)_2D_3$*

3 min incubation with 1,25-$(OH)_2D_3$ (100 nM) increased intracellular cGMP to 215 ± 20% of control. Preincubation with actinomycin D for 30 min completely abolished this effect (fig.1). The same pretreatment did not affect sodium nitroprusside stimulation of cGMP. Preincubation with α-amanitin decreased the stimulatory effect of 1,25-$(OH)_2D_3$ on cGMP ($P < 0.001$) by 50% (fig.1). The same treatment did not influence the sodium nitroprusside effect on cGMP. Actinomycin D or α-amanitin treatment without added hormone increased the baseline intracellular cGMP concentration (165 ± 15 and 133 ± 5%, respectively; $P < 0.005$ and 0.01).

30 min incubation with actinomycin D inhibited RNA synthesis down to 21 ± 5% of control (mean of 3 experiments, each with 8 replicates). Similar treatment with α-amanitin inhibited RNA synthesis down to 54 ± 12% (mean of two experiments, each with 8 replicates).

3.2. *Effects of protein synthesis inhibitors on the rapid cGMP rise caused by steroids*

In these experiments 1,25-$(OH)_2D_3$ increased intracellular cGMP concentration to 174 ± 18 and 188 ± 16% of control. Preincubation with cycloheximide completely abolished this action (fig.2). The same preincubation affected neither the baseline cGMP value, nor the sodium nitroprusside effect. Preincubation with diphtheria toxin also inhibited the ability of 1,25-$(OH)_2D_3$ to increase cGMP (fig.2). Diphtheria toxin alone decreased the baseline cGMP concentration (to 71

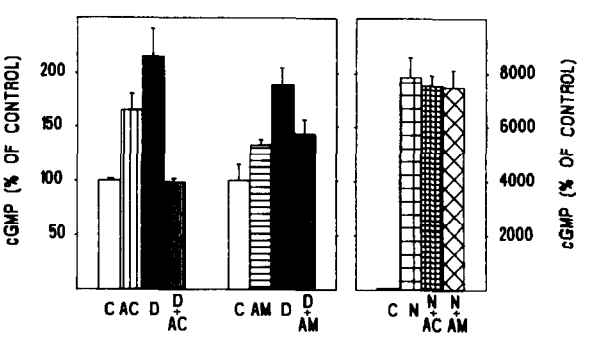

Fig.1. Effects of RNA synthesis inhibitors (AC, actinomycin D; AM, α-amanitin) on 1,25-$(OH)_2D_3$ (D) and sodium nitroprusside (N) mediated increase of cGMP. Inhibitors were present during a 30 min preincubation; cGMP agonists were present for 3 min. Bars represent mean ± 1 SE of 3 experiments. Control cGMP value (C) was 70 ± 4 pmol/mg protein.

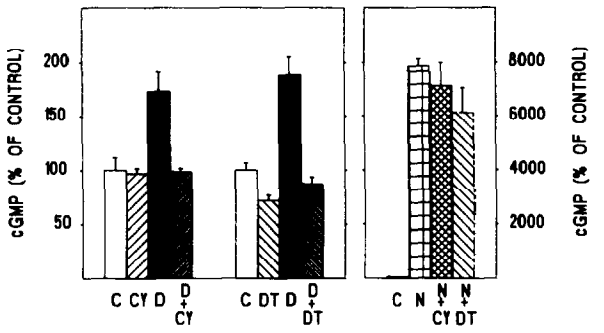

Fig.2. Effects of protein synthesis inhibitors (Cy, cycloheximide; DT, diphtheria toxin) on 1,25-$(OH)_2D_3$ (D) mediated increase of cGMP. Inhibitors were present during a 30 min preincubation; cGMP agonists were present for 3 min. Bars represent mean ± 1 SE of 2 experiments. Control cGMP value (C) was 58 ± 5 pmol/mg protein.

± 5%, $P < 0.01$) and slightly decreased the sodium nitroprusside effect (to 78 ± 7%, $P < 0.01$).

Cycloheximide or diphtheria toxin prevented the rapid stimulation of cGMP by testosterone or dexamethasone (table 1).

3.3. *Effect of actinomycin D on 1,25-$(OH)_2D_3$ binding*

30 min incubation of fibroblasts with actinomycin D did not change 1,25-$(OH)_2D_3$-binding capacity (2650 vs 2680 binding sites/cell with and without actinomycin D) or binding affinity (K_d = 0.39 vs 0.34 nM).

Table 1

Effects of protein synthesis inhibitors on the stimulation of intracellular cGMP by agonists

cGMP regulator	cGMP	cGMP after cycloheximide	cGMP after diphtheria toxin
None	100 ± 2	97 ± 12	76 ± 3[a]
Testosterone (100 nM)	148 ± 7	83 ± 9[b]	79 ± 7[b]
Dexamethasone (100 nM)	189 ± 8	113 ± 6[b]	76 ± 7[b]
Na nitroprusside (1 mM)	5460 ± 320	5380 ± 96	5000 ± 172[a]

[a] $P < 0.01$ for with vs without metabolic inhibitor
[b] $P < 0.001$ for with vs without metabolic inhibitor

Inhibitors of protein synthesis were preincubated with cells for 30 min and incubation with cGMP agonists was for 4 min. Results (expressed as % of control) represent mean ± 1 SE of 2 experiments. Control cGMP concentration was 172 ± 11 pmol/mg protein

4. DISCUSSION

We found that each of four metabolic inhibitors prevented the rapid stimulatory action of 1,25-$(OH)_2D_3$ on intracellular cGMP. Actinomycin D and α-amanitin inhibited this rapid action of 1,25-$(OH)_2D_3$ at concentrations that effectively blocked RNA synthesis. It is unlikely that these effects we observed with RNA synthesis inhibitors reflect general depression of cGMP metabolism, since, under our conditions of brief preincubation with metabolic inhibitors, these inhibitors did not prevent the rise of cGMP caused by sodium nitroprusside. Actinomycin D or α-amanitin in fact stimulated cGMP, perhaps through generation of oxygen free radicals [12].

We have shown here also that 30 min preincubation with inhibitors of protein synthesis (cycloheximide or diphtheria toxin) blocked the 1,25-$(OH)_2D_3$-mediated rise in cGMP. This inhibitory effect is also unlikely to be the result of toxic effect on cGMP metabolism, because cGMP could still be increased by sodium nitroprusside. Since all four metabolic inhibitors prevented the cGMP rise by 1,25-$(OH)_2D_3$, and since the only common effect of these chemicals is the direct or indirect inhibition of protein synthesis, it is highly likely that inhibition of protein synthesis (and not some other 'nonspecific' effects of these metabolic inhibitors) accounts for their selective effects on 1,25-$(OH)_2D_3$ regulation of cGMP.

Is it likely that the 1,25-$(OH)_2D_3$ receptor itself is the rapidly turning over protein affected by the metabolic inhibitors in our study? Actinomycin D inhibited 1,25-$(OH)_2D_3$ receptor concentration during 20 h induction of receptor in kidney cells (LLC-PK1) [13]. Sher et al. [14] reported generally inhibitory effects on receptor concentration in human breast cancer cells (T47D) after 2–8 h incubation with inhibitors of transcription or of protein synthesis. Since the 1,25-$(OH)_2D_3$ receptor has a half-life of approx. 4 h, we used only a brief exposure to actinomycin D (and other metabolic inhibitors); with this brief preincubation, we did not find detectable effect on receptor number or affinity. It seems unlikely that the receptor itself is the metabolic inhibitor-sensitive protein needed for

hormonal stimulation of cGMP; however, we cannot exclude the possibility that a small pool of newly synthesized receptor is implicated.

Our findings can be explained in one of two ways: (i) 1,25-$(OH)_2D_3$ acts through modulation of mRNA synthesis very rapidly thereby changing concentration of a cGMP-regulating protein, or (ii) a rapidly turning over message encodes a rapidly turning over protein, which is necessary for a fast action of 1,25-$(OH)_2D_3$ on cGMP. The first explanation is unlikely because the 1 min activation time is not sufficient for induction of de novo protein synthesis. There was a prior suggestion for the involvement of a rapidly turning over protein in the slow 1,25-$(OH)_2D_3$ effect to increase vitamin D-dependent calcium-binding protein mRNA synthesis in chick intestine, but a toxic effect of cycloheximide on total RNA synthesis was not excluded [15].

The requirement for ancillary protein in this 1,25-$(OH)_2D_3$ receptor-mediated rapid increase of cGMP suggests a mechanism whereby the receptor could interact with an effector other than its putative DNA recognition element. The ancillary protein(s) could interact directly with the receptor, the effector, or both. A similar intermediary role has been suggested in glucocorticoid receptor induction of the rat α-acid glycoprotein gene; the promoter region of that gene lacks a consensus glucocorticoid recognition element, and the intermediary protein may allow receptor-promoter interaction [3].

Rapidly turning over protein(s) has not been previously implicated in fast actions of 1,25-$(OH)_2D_3$ or other steroids. Our results indicate that the requirement for ongoing RNA and protein synthesis is not limited to 1,25-$(OH)_2D_3$ effects with a long lag period, and that such a requirement need not imply a genomic 1,25-$(OH)_2D_3$ action. Induction of spermidine N_1-acetyltransferase occurs within 30 min of 1,25-$(OH)_2D_3$ treatment, and actinomycin D or cycloheximide blocked this action [16]. This was interpreted as evidence for the involvement of extremely rapid de novo RNA and protein synthesis [11], but our findings indicate that rapidly turning over protein(s) with or without a genomic 1,25-$(OH)_2D_3$ action are alternative possibilities.

We showed previously that a rapid rise of cGMP in fibroblasts can be caused not only by 1,25-$(OH)_2D_3$ but also by androgens [10]. Now we have found that a rapid cGMP rise can also be caused by dexamethasone. This rapid rise of cGMP caused by testosterone or dexamethasone, like that caused by 1,25-$(OH)_2D_3$, was also blocked by 30 min preincubation with protein synthesis inhibitors. The similar effects of 1,25-$(OH)_2D_3$ and other steroids on cGMP and the similar dependency of all these steroid hormones on rapid protein turnover suggest involvement of a common process interacting with their receptors. The labile protein or proteins involved in one rapid action of the 1,25-$(OH)_2D_3$ receptor might not only be related to proteins mediating rapid effects of other steroids but also to proteins implicated in less rapid actions of these hormones [2–5].

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