THE EFFECT OF N^6-2'-O-DIBUTYRYL-ADENOSINE 3',5'-MONOPHOSPHATE ON RAT LIVER CALCIFEROL 25-HYDROXYLASE

Sorel Sulimovici and Martin S. Roginsky

Department of Medicine, Nassau County Medical Center
East Meadow, New York
State University of New York at Stony Brook

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SUMMARY. Liver calciferol 25-hydroxylase activity of vitamin-D deficient rats was enhanced 24 hours following the intravenous injection of N^6-2'-O-dibutyryl adenosine 3',5'-monophosphate. Sodium butyrate administered in the same way had no effect on this enzyme system. Administration of actinomycin D with N^6-2'-O-dibutyryl adenosine 3',5'-monophosphate abolished the stimulatory effect of the cyclic nucleotide. Direct addition to the incubation medium of adenosine 3',5'-cyclic monophosphate or of its dibutyryl derivative did not influence the hepatic conversion of cholecalciferol to 25-hydroxycholecalciferol. These results suggest a possible role for the cyclic nucleotide in the regulation of this enzyme system.

It has been established that the conversion of cholecalciferol (vitamin D_3) to more biologically active forms requires two successive hydroxylations. First, 25-hydroxylation leading to the formation 25-hydroxycholecalciferol (25-OH-vit. D_3), produced mainly by the liver (1) and subsequent hydroxylation by the kidney of 25-OH-vit. D_3 to 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$-vit. D_3) (2). Calciferol-25-hydroxylase, the enzyme involved in the initial hydroxylation of vitamin D_3 has been demonstrated in liver of the vitamin D deficient chick (3) and rat (1,4). It was suggested that this liver enzyme is feed-back regulated by the product (25-OH-vit. D_3) and by the vitamin D state of the animal (5). The kidney enzyme, 1-hydroxylase, was shown to be controlled by the intracellular concentrations of calcium; an effect mediated through the action of parathyroid hormone (PTH) and cyclic AMP (6,7). No hormonal control has been implicated for liver calciferol-25-hydroxylase and the effect of cyclic cAMP on this enzyme has not been investigated. Since cyclic nucleotide has been found to influence the activity of other liver enzymes (8), it was decided to investigate the effect of cyclic AMP on the

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hydroxylation of vitamin D_3 by the liver. The results of these experiments demonstrate that N^6-2'-O-dibutyryl adenosine 3',5'-monophosphate (dibutyryl-cAMP) injected intravenously to vitamin-D deficient rats stimulates liver calciferol-25-hydroxylase.

Materials and Methods

The radioactive compounds (1α, 2α (n)-^{3}H) vitamin D_3 (spec. activ. 8.2 Ci/mmol) and 25-hydroxy-vitamin D_3 (26-(27) methyl-^{3}H) (spec. activ. 2-9 Ci/mmol) were obtained from Amersham Searle Corporation. Dibutyryl-cAMP sodium butyrate and actinomycin D were from Sigma. Other chemicals and organic solvents were of analytical reagent grade.

Male albino rats (Holtzman Co., Madison, Wisconsin) were used in these experiments. The rats were fed on a low vitamin-D diet (Nutritional Biochemicals, Cleveland, Ohio) and water supplemented ad libitum. The experiments were carried out on animals maintained on this regimen for 4-6 weeks. Dibutyryl-cAMP or sodium butyrate, 10 mg per 100 g body weight in 0.1 ml of 0.9% NaCl was injected intravenously. Control subjects received only 0.1 ml of 0.9% NaCl. When injected, actinomycin D 75 µg per 100 g body weight was dissolved in 50% ethanol, brought to 0.1 ml volume with 0.9% NaCl and administered intraperitoneally. The rats were killed by a sharp blow in the head and the liver removed and homogenized in ice-cold 0.25 M Sucrose. A fraction of liver homogenate corresponding to 1g wet tissue was incubated in the presence of 0.1 M K_2HPO_4 buffer (pH 7.4), $MgSO_4$ 5.0 mM, KCl 0.1 M, nicotinamide 0.16 M, ATP 20 mM, $NADP^+$ 0.4 mM, glucose-6-phosphate 22 mM and 0.2 µCi (^{3}H) vitamin D_3 (25 pmoles) solved in a small amount of ethanol. The total volume was 3 ml and the incubations were carried out in air for 2h at 37°C. The incubation period was terminated by the addition of 0.2 ml acetic acid and freezing. The mixture was extracted three times with 15 ml ethyl acetate, and the combined extracts washed with distilled water (5 ml) dried by anhydrous sodium sulphate and evaporated to dryness under nitrogen and vacuum. Using this technique, 90 percent of the added radioactive

vitamin D_3 was recovered. Separation of the extracted sterols was carried out on a 1x30 cm column of 17g of sephadex LH-20 following the procedure described by Holick and DeLuca (9). Substrate tritiated vitamin D_3 only, was also added to buffer and liver homogenate samples and carried through the entire procedure. Enzymic activity, expressed as pmoles 25-OH-vit. D_3 formed per g. liver tissue, was calculated from the data obtained from the conversion of added (^{3}H) vitamin D_3 to (^{3}H) 25-OH-vit. D_3. Chromatography on silicic acid was carried out after the technique of Preece et al. (10) while liquid-liquid partition chromatography followed the procedure of Blunt et al. (11). The vitamin-D binding protein was prepared from serum of vitamin D-deficient rat (12).

Results

The identity of (^{3}H) 25-OH-vit. D_3 eluted from the sephadex LH-20 column was confirmed by silicic acid chromatography and by celite liquid-liquid partition chromatography. Employing these latter procedures, the compound isolated

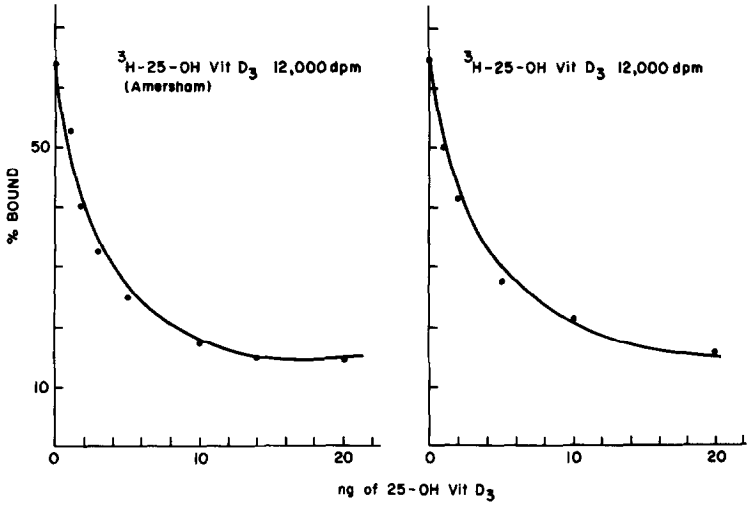

Figure 1. Standard curves of (^{3}H) 25-OH-Vit. D_3 obtained from Amersham and that eluted from Sephadex LH-20 columns. Percent bound (^{3}H) 25-OH-Vit. D_3 is plotted as a function of the amount of unlabelled 25-OH-Vit. D_3. Each point represents the mean of three replicate determinations. Dilution of the vitamin D-binding protein was 1/1600.

TABLE I. Effect of dibutyryl-cAMP and sodium butyrate on rat liver calciferol 25-hydroxylase activity*.

	Number of Animals	Number of Replicates	25-OH-Vit. D_3 pmoles/g liver (mean ± SEM)	Significance** (Versus Control)
Experiment 1				
CONTROL	(5)	5	3.1 ± 0.15	
Dibutyryl-cAMP	(5)	5	5.2 ± 0.17	$p < 0.001$
Experiment 2				
CONTROL	(6)	5	2.7 ± 0.12	
Dibutyryl-cAMP	(6)	5	4.3 ± 0.14	$p < 0.001$
Experiment 3				
CONTROL	(5)	5	3.6 ± 0.15	
Dibutyryl-cAMP	(6)	5	6.3 ± 0.18	$p < 0.001$
Experiment 4				
CONTROL	(5)	5	3.4 ± 0.16	
Dibutyryl-cAMP	(5)	5	7.2 ± 0.36	$p < 0.001$
Experiment 5				
CONTROL	(5)	4	3.0 ± 0.14	
Sodium Butyrate	(5)	4	3.4 ± 0.16	NS

* Twenty-four hours before assay rickitic rats received a single intravenous injection of either dibutyryl-cAMP or sodium butyrate 10 mg/100 g body weight suspended in 0.1 ml 0.9% saline. Control animals received 0.1 ml saline only. Calciferol 25-hydroxylase activity was measured as described under the section Methods.

** Statistical significance was determined by the student t test.

corresponded to standard 25-OH-vitamin D_3. To further establish the identity of this compound, the binding of (^{3}H) 25-OH-vit. D_3 eluted from the sephadex LH-20 column to the vitamin-D transport protein isolated from rat serum was compared with (^{3}H) 25-OH-vit. D_3 obtained from Amersham Corporation (Fig. 1). It can be seen, that increasing amounts of cold 25-OH-vit. D_3 produced identical displacement of (^{3}H) 25-OH-vit. D_3 obtained from Amersham or that eluted from the sephadex LH-20 column following the incubation of liver homogenates with (^{3}H) vitamin D_3 and an NADPH generating system.

The effect of dibutyryl-cAMP and sodium butyrate injected intravenously to vitamin-D deficient rats on liver calciferol 25-hydroxylase is shown in Table 1. The enzymic activity was significantly increased in the dibutyryl cAMP injected animals by comparison with the control group. Similar results were obtained when dibutyryl-cAMP was administered intraperitoneally and in both cases a response was observed when the animals were sacrificed 24-hours after the injection. Sodium butyrate had no effect on the conversion of (^{3}H) vitamin D_3 to (^{3}H) 25-OH-vit. D_3 (Table 1, Experiment #5). Direct addition to the incubation

TABLE 2. Effect of dibutyryl-cAMP with and without actinomycin D* on rat liver calciferol 25-hydroxylase activity.

	25-OH-Vit. D_3 pmoles/g liver** Without Actinomycin D	With Actinomycin D
Control (Saline)	2.4 ± 0.15 (5)	2.8 ± 0.14 (5)
Dibutyryl-cAMP	4.4 ± 0.17 (5)***	2.4 ± 0.11 (5)

* Actinomycin D 75 µg/100 g body weight was administered intraperitoneally, followed immediately by the intravenous injection of dibutyryl-cAMP (10 mg/100 g body weight). The rats were sacrificed 24-hours later.

** Livers from each group (5-6 animals) were pooled, homogenized and tested for calciferol 25-hydroxylase activity as described under the section on Methods. Results are given as the mean ± SE of the mean of five separated incubation.

*** When compared with control $p < 0.001$.

medium of cyclic AMP or its dibutyryl derivative did not affect 25-hydroxylation of vitamin D_3. When rats were given simultaneous injections of dibutyryl-cAMP and actinomycin D, the effect of this cyclic nucleotide on calciferol 25-hydroxylase activity was abolished (Table 2). When actinomycin D was administered alone, no effect on the 25-hydroxylase activity was observed.

Discussion

Calciferol 25-hydroxylase activity measured by the in-vitro conversion of (^{3}H) vitamin D_3 to (^{3}H) 25-OH-vit. D_3 was found to be present in liver homogenates prepared from vitamin-D deficient rats. These results are in good agreement with previous findings which established the presence of this enzyme in rat liver homogenates (1,5). The present study demonstrates that dibutyryl-cAMP injected intravenously to rickitic rats increases liver calciferol 25-hydroxylase activity. Exogenous administration of dibutyryl-cAMP has been shown to cause an increase in the synthesis or activity of many hepatic enzymes such as: ornitine decarboxylase (13), serine dehydratase (14), phosphopyrurate carboxylase (15), and tyrosine a-ketoglutarate transaminase (16,17). The data presented in Table 2 demonstrate that actinomycin D injected simultaneously with dibutyryl-cAMP inhibited the stimulation of calciferol 25-hydroxylase by the nucleotide. This suggests that the increased activity of the 25-hydroxylation system in response to dibutyryl-cAMP may depend upon new messenger RNA formation. Comparable results were reported for liver ornitine decarboxylase (13) and serine dehydratase (14) where, actinomycin D inhibited the stimulation of these enzymes by dibutyryl-cAMP. On the other hand, experiments carried out in-vitro have shown that actinomycin D does not prevent the stimulation of 1-hydroxylase by dibutyryl-cAMP indicating that this action of the cyclic nucleotide on this vitamin D metabolite is independent of new protein synthesis (18). In the present experiments, dibutyryl-cAMP added in-vitro to the liver homogenates had no effect on calciferol 25-hydroxylase activity. This suggests a requirement for new protein synthesis in the mechanism of activity of cyclic nucleotide on 25-hydroxylase.

Intraperitoneal injection of the antibiotic to rickitic rat did not impair 25-hydroxylase activity (Table 2). This is in accord with the reported results for the action of actinomycin D on 1-hydroxylation (19) when administered in-vivo to the chick and differs from the observation in which administration of the antibiotic to the rat inhibited the 1-hydroxylase enzyme (20,21).

The results of the present experiments suggest that cyclic AMP may be involved in the regulation of liver calciferol 25-hydroxylase system, probably through the formation of new messenger RNA.

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