A "SECOND MESSENGER" FOR VITAMIN D

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1. Introduction

Vitamin D_3 can be considered to be a hormone produced by the skin, stored in the liver, and released from there to act at a variety of sites in the body in particular at the small intestine to aid the absorption of calcium [1]. Many hormones are considered to function via a "second messenger" [2] usually cyclic 3′,5′-adenosine monophosphate (cyclic AMP) and it is of interest to know whether vitamin D_3 (hormone) also uses this second messenger during its effect on calcium and phosphate metabolism. This letter reports the effect of vitamin D_3 on adenyl cyclase and on the effect of a derivative of cyclic AMP on calcium transport across the small intestine of the chick.

2. Experimental procedure

2.1. *Chickens*

White crossbred cockerals were obtained on the day of hatching and reared for 4 weeks on a diet deficient in vitamin D and selected as deficient as previously described [3]. To study the effect of vitamin D, chicks were given 400 I.U. intramuscularly 16 hr, or 5000 I.U. in 0.1 ml propylene glycol 3 hr before the experiments.

2.2. *Measurement of adenyl cyclase*

Mucosal cells were scraped from the washed lower half of the small intestine of three chickens, the pooled "membrane fraction" prepared and its adenyl cyclase activity measured as described by Streeto and Reddy [4]. The incubation mixture was modified to include myokinase 10 μg/ml, and during the 15 m incubation with the "membrane fraction" the ^{14}C ATP was added in two portions at zero and 7 min using a total of 2.5 μc and 1 μmol of ATP. The deproteinized incubation mixture was chromatographed in *n*-propanol, conc. ammonia, water (60, 30, 10, v/v) and the zone corresponding to cyclic AMP was eluted from the paper, concentrated to small volume and rechromatographed in the ethanol, boric acid solvent of Streeto and Reddy [4]. The cyclic AMP was eluted, concentrated to dryness in a scintillation vial and counted by scintillation counting. The radioactivity of the ^{14}C cyclic AMP was corrected for loss of material during the above procedures by observing the recovery of the ^{3}H cyclic AMP which was added at the end of incubation [4]. When prostaglandin E_1 was included in the incubation the concentration was 1.8×10^{-5} M.

2.3. *Measurement of calcium absorption in vivo*

Closed sacs of ileum were prepared as described by Coates and Holdsworth [5] and 1 mg $^{45}CaCl_2$ (1–5 μc) in 0.5 ml injected. The bird was killed 20 min later; the amount of ^{45}Ca remaining in the gut, circulating in the blood and deposited in the tibia was determined as described by Neville and Holdsworth [6]. To test the effect of cyclic AMP on absorption of Ca^{++}, the N^6, 2-*O*-dibutyryl-adenosine-3′,5′-cyclic monophosphate (C.F.Boehringer, Mannheim) derivative was used and 4 mg in 0.1 ml saline was injected intraperitoneally into a 150 ± 10 g chick at various times before placing the ^{45}Ca in the ileum.

2.4. *Calcium translocation by everted sacs*

Sacs of isolated everted ileum were prepared as previously described [3]. The mucosal and serosal

fluids contained initially 5×10^{-4} M Ca^{++} and where appropriate 1×10^{-3} M dibutyryl cyclic AMP. The mucosal fluid contained 1 μc/ml ^{45}Ca, and the amount of ^{45}Ca accumulated in the serosal fluid during 30 min incubation at 35° was measured [6].

3. Results

3.1. *The effect of vitamin D_3 and prostaglandin on adenyl cyclase*

The "membrane fraction" from vitamin D-deficient chick intestine catalysed the formation of small amounts of cyclic AMP from ATP, in the presence of caffeine which is believed to inhibit phosphodiesterase. The amount of cyclic AMP was increased by pretreatment with vitamin D_3 even when the vitamin was given only 3 hr before the experiment (table 1) and in four similar experiments the ratio of treated (16 hr D_3) to untreated was 1.51 ± 0.16. These results suggest that the amount or the activity of adenyl cyclase is increased by vitamin D_3 treatment.

The addition of prostaglandin E_1 to the incubation mixture increased the yield of cyclic AMP from the vitamin D_3-deficient preparation and from the preparation from the birds given vitamin D 3 hr previously. However, it had little effect on the preparation that had received 400 I.U. of vitamin D_3 16 hr previously.

Table 1

Measurement of adenyl cyclase activity. 0.4 ml incubation mixture contained 4 mg protein and 1.0 μmol (2.5 μc) ^{14}C ATP at 30° for 15 min.

Pretreatment	μμmole ^{14}C cyclic AMP/mg protein/15 min	
		in the presence of prostaglandin E_1
None	281	372
5000 I.U. vitamin D_3 intraperitoneally 3 hr previously	520	684
400 I.U. vitamin D_3 intramuscularly 16 hr previously	352	376

3.2. *The effect of dibutyryl cyclic AMP on calcium absorption in vivo*

As previously shown, pretreatment with vitamin D_3, 16 hr previously, increased calcium absorption from the ileum [1,3,5,6] and appearance of ^{45}Ca in blood and bone. Three experiments were done in which birds were pretreated with dibutyryl cyclic AMP at the following dose levels: (i) 4 mg at each of 20 min, 10 min and zero (table 2), (ii) 4 mg at each of 20 min and 10 min, (iii) 4 mg at 10 min before the ^{45}Ca dose was given. In all three experiments the ^{45}Ca absorption and incorporation into bone was increased. An analysis of variance of the combined results with the different dose levels showed that the effect of dibutyryl cyclic AMP was significant for incorporation into bone ($P < 0.1$) and for absorption from the gut ($P < 0.02$).

Table 2

The effect of dibutyryl cyclic AMP on calcium absorption *in vivo*. 4 mg of dibutyryl cyclic AMP in 0.1 ml 0.15 M NaCl was injected intraperitoneally at each of 20 min, 10 min and zero before placing 1 mg ^{45}Ca into ileal sacs *in vivo* (4 birds in each group).

Pretreatment	Plasma (cpm/ml)		Bones (cpm/tibia)	Ca absorbed (cpm × 10^{-3})
	10 min	20 min		
None	2,380 ± 788	3,320 ± 944	6,794 ± 2,742	1,136 ± 799
Dibutyryl cyclic AMP	3,790 ± 1,500 *	5,390 ± 2,160 **	9,987 ± 2,386 **	2,857 ± 994 **
D_3 400 I.U. 16 hr previously	18,460 ± 4,680	23,830 ± 3,910	38,072 ± 11,575	4,770 ± 2,460

* Significantly greater than untreated rachitic control $P = 0.2$.
** Significantly greater than untreated rachitic control $P < 0.2$.
*** Significantly greater than untreated rachitic control $P < 0.05$.

Table 3
The effect of dibutyryl cyclic AMP on calcium translocation by everted sacs. Each result is the mean of 3 sacs prepared as described in section 2.

Pretreatment of rachitic chicks	Composition of mucosal and serosal fluids	^{45}Ca cpm in serosal fluid after 30 min
None	Ringer	10,200 ± 843
None	Ringer + 10^{-3} M dibutyryl cyclic AMP	14,970 ± 3,366 **
Vitamin D 400 I.U. 16 hr previously	Ringer	29,400 ± 1,616
Vitamin D 400 I.U. 16 hr previously	Ringer + 10^{-3} M dibutyryl cyclic AMP	27,990 ± 2,228 **

* Significantly greater than the control without dibutyryl cyclic AMP, $P < 0.2$.
** Not significantly greater than the vitamin-treated sacs without dibutyryl cyclic AMP, $P > 0.6$.

3.3. *The effect of dibutyryl cyclic AMP in vitro on calcium translocation in everted sacs*

Addition of dibutyryl cyclic AMP to the fluid bathing both sides of the everted ileum appeared to increase calcium translocation by gut from rachitic chicks ($P < 0.2$), whereas it appeared to have no significant effect on calcium translocation by the gut of chicks that had been pretreated with vitamin D_3 ($P > 0.6$) (table 3).

4. Discussion

The time lag between the administration of vitamin D and the observed increase in calcium absorption is 5–8 hr [7,3], which has been shortered to 3 hr by using the metabolite 25 hydroxycholecalciferol intravenously [8]. However, it is known that vitamin D increases incorporation of uracil into what is presumed to be messenger RNA of mucosal cells within 30 min. There has been no suggestion as to what new proteins are coded by this increased amount of messenger [7].

In this paper it has been shown that a large dose of vitamin D caused a doubling of adenyl cyclase activity within 3 hr. Thus vitamin D may be added to the increasing number of hormones thought to act via a "second messenger" [2]. If this is so then cyclic AMP should cause a rapid stimulation of calcium absorption. This was tested *in vivo* and *in vitro* by using the dibutyryl derivative of cyclic AMP which is presumed to be able to pass across the cell membrane more readily than cyclic AMP. Amounts of 4 to 12 mg of the cyclic AMP derivative were given to 150 g chicks and within 10–20 min had caused an increased absorption of calcium from the gut of rachitic chicks, and increased ^{45}Ca in blood and in bone. The effect was also demonstrated even when dibutyryl cyclic AMP was added to tissue *in vitro* and thus could not be exerting its effect by changing blood flow, clearance of calcium from the serosal surfaces, or deposition in bone. When added to everted sacs of ileum from rachitic chicks, dibutyryl cyclic AMP caused an increased translocation of ^{45}Ca to the serosal fluid, but no such effect was observed on the gut from chicks replete with vitamin D_3. This is what would be expected if cyclic AMP is concerned in the effect of vitamin D on calcium transport.

It has been pointed out that cyclic AMP and prostaglandins are ubiquitous in mammalian cells although the exact relationship between these substances is unknown [9]. It was interesting to find that physiological levels of prostaglandin E_1 when included in the incubation mixture for measuring adenyl cyclase caused an increased yield of cyclic AMP. The effect was similar to that obtained by injection of the chick with vitamin D. Prostaglandin, however, enhanced the yield of cyclic AMP even when vitamin D-replete chicks were used. The mechanism whereby prostaglandin appears to increase adenyl cyclase activity is unknown and there has been no published information on the effect of prostaglandins on the permeability of the gut to calcium. This is currently being investigated.

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