Oxygen-dependent 1,25-dihydroxycholecalciferol-induced calcium ion transport in rat intestine

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O₂-dependent Ca²⁺ uptake by rat duodenal discs has been characterized and used in a revised assay for 1,25-dihydroxycholecalciferol-induced intestinal Ca²⁺ transport. Although both muscle and mucosal surfaces are exposed in this free-floating-disc assay, the Ca²⁺ influx across the muscle surface is small, not O₂- or vitamin D-dependent, and can be subtracted out. Depriving the animals of food for 9–14 h before assay increases the O₂-dependent uptake by about 75%. Half-saturation values for O₂-dependent Ca²⁺ uptake as determined with this assay are: 0.8 mM-Ca²⁺ (fed) and 0.5 mM-Ca²⁺ (food-deprived) for vitamin D-deficient rats, and 0.9 mM-Ca²⁺ (fed) and 1.5 mM-Ca²⁺ (food-deprived) for rats dosed with 1,25-dihydroxycholecalciferol. The maximum velocity of uptake varies from 6.7 nmol of Ca²⁺ per cm²/min (fed) to 7.0 nmol of Ca²⁺ per cm²/min (food-deprived) for vitamin D-deficient rats and 16.7 nmol of Ca²⁺ per cm²/min (fed) to 29 nmol of Ca²⁺ per cm²/min (food-deprived) for 1,25-dihydroxycholecalciferol-treated rats. By using a 5 min preincubation and 15 min incubation with 1.0 mM-Ca²⁺, duodenal tissue taken from vitamin D-treated rats shows about a 3-fold increase in O₂-dependent Ca²⁺ uptake when compared with tissue taken from vitamin D-deficient animals. The calcium ionophore A23187, depending on concentration, either has no significant effect on or inhibits the O₂-dependent uptake, rather than increasing it. Actinomycin D, at a dose of 2 µg/g, inhibits the O₂-dependent uptake in intestinal discs from both vitamin D-deficient and vitamin D-treated rats by 58 and 80% respectively, when administered *in vivo* 3½ h before assay.

Numerous studies on vitamin D-dependent intestinal Ca²⁺ transport, utilizing many techniques, have appeared in the past two decades, each contributing new facts about this hormone-induced process. In the rat, the duodenal transport has been shown to be an active, O₂-dependent flux in the mucosal-to-serosal direction, with a half-saturation point of 1–2 mM-Ca²⁺ (for reviews, see DeLuca, 1978; Wasserman & Taylor, 1976). It has been shown that as little as 25 ng of 1,25(OH)₂D₃, the active metabolite of vitamin D, will increase duodenal Ca²⁺ transport of a vitamin D-deficient rat by a factor of two in about 6 h; this increase persists for several days (Napoli *et al.*, 1978).

However, several basic questions concerning the cellular and molecular nature of the vitamin-induced

process are still controversial. For example, the 1,25(OH)₂D₃-induced transport step has been suggested to occur at the brush border (Adams *et al.*, 1970; Papworth & Patrick, 1970), intracellularly (Freedman *et al.*, 1977), and at the basal-lateral surface (Schachter *et al.*, 1966). It is not certain whether the hormone acts through synthesis of new proteins, as might be expected of such a steroid-like molecule with high-affinity intestinal receptors (Kream *et al.*, 1977; Wilson & Lawson, 1977), or whether it works totally differently, e.g. by somehow changing the lipid composition of the brush-border membrane and thus Ca²⁺ permeability (Rasmussen *et al.*, 1979).

Most of the techniques used to measure intestinal Ca²⁺ flux have some inherent flaw that interferes with precise quantification of the O₂-dependent hormone-induced response. The everted-gut-sac technique of Wilson & Wiseman (1954), often used to measure intestinal Ca²⁺ transport, requires at least a 90 min incubation time to achieve Ca²⁺

Abbreviations used: 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; 25(OH)D₃, 25-hydroxycholecalciferol; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

equilibration across the intestinal muscle layer. This is probably in excess of the time during which the villus layer remains intact (Levine *et al.*, 1970). Also, the maximum serosal/mucosal Ca^{2+} gradient that may be maintained across the intestinal layers at equilibrium may be a limiting factor in the everted-gut-sac assay.

Methods designed to measure transintestinal fluxes of Ca^{2+} , using a Ussing-type apparatus, suffer the same problems of muscle impermeability and resultant long incubation times, although large Ca^{2+} gradients are not generated with these methods, and transmural potential differences may be nulled. These methods are most useful for measuring serosal $\rightarrow$ mucosal and mucosal $\rightarrow$ serosal ion fluxes in fewer animals (Walling & Rothman, 1969; Walling *et al.*, 1974).

Methods *in situ* and *in vivo* used to measure intestinal Ca^{2+} flux, although physiological, do not separate out the active hormone-induced transport from passive diffusion. The latter is a large component of total Ca^{2+} transport and may obscure the hormone effect.

With these considerations in mind, a revised assay for intestinal Ca^{2+} transport has been devised based on the $^{45}\text{Ca}^{2+}$ -uptake studies of Schachter *et al.* (1960, 1961), Martin & DeLuca (1969) and Papworth & Patrick (1970). In the revised assay, the $^{45}\text{Ca}^{2+}$ uptake into intestinal discs of known area has been measured after removal of externally bound Ca^{2+} and found to be linear for about 15 min. The uptake into discs incubated under N_2 from 1,25(OH) $_2\text{D}_3$ -treated rats is virtually identical with that into discs under N_2 from vitamin D-deficient animals. This uptake is roughly 30% of the uptake found in discs from rats given 1,25(OH) $_2\text{D}_3$ and incubated under O_2 . Thus a comparison of O_2 -dependent $^{45}\text{Ca}^{2+}$ uptake between 1,25(OH) $_2\text{D}_3$ -dosed and cholecalciferol-deficient animals can be obtained by subtraction of uptake under N_2 from uptake under O_2 . The revised assay has been used to examine the effects of actinomycin D and ionophore A23187 upon the rat intestinal active Ca^{2+} -transport process.

Experimental

Young male Holtzman rats, raised on a vitamin D-deficient diet containing 0.5% Ca^{2+} and 0.3% phosphorus (Suda *et al.*, 1970), were used in all experiments. The rats were obtained as weanlings and housed in hanging wire cages for 4.5–6 weeks before use. Rats raised on an identical diet were observed to have undetectable amounts ($<5\text{ pg/ml}$) of 25(OH) D_3 and 1,25(OH) $_2\text{D}_3$ in serum after 3–4 weeks.

Unless otherwise noted in the text, all animals used were dosed intrajugularly under light diethyl

ether anaesthesia with 125 ng of 1,25(OH) $_2\text{D}_3$ in 50 μl of ethanol or with ethanol alone, 24 h before being killed.

The Ca^{2+} ionophore was generously provided by Dr. Robert Hammell of the Lilly Research Laboratories, Indianapolis, IN, U.S.A. Actinomycin D was purchased from Sigma. All other chemicals were of Reagent grade.

Free-floating-disc assay

The animals were killed by decapitation, and about 6 cm of proximal duodenum was removed and gently rinsed with 5 ml of ice-cold 150 mM-NaCl. The duodenum was opened on to a hardwood block and two discs were cut out with a cork borer of convenient size from within 3 cm of the proximal end. Initially the discs were cut out on an iced block to minimize curling of the intestine; later smaller discs were taken from tissue opened on to a warm block. This seemed to increase uniformity of muscle contraction and intestinal thickness, variation in the measurement of Ca^{2+} uptake being decreased. The area of the discs varied from 0.38 cm^2 to 0.785 cm^2 ; all uptake values were normalized to 1 cm^2 . The most proximal disc was placed in a 25 ml Erlenmeyer flask containing 9 ml of 140 mM-NaCl, 22.2 mM-fructose and 10 mM-Hepes, pH 7.4, flushed with O_2 and warmed to 37°C. The distal disc was placed into a similar solution without fructose and flushed with N_2 , also at 37°C. After 5 min preincubation with shaking, 1.0 ml of a prewarmed solution of $\text{Ca}^{2+} + ^{45}\text{Ca}^{2+}$, 140 mM-NaCl and 10 mM-Hepes, pH 7.4, was added to each flask and the incubation continued for the appropriate time, usually 15 min. The specific radioactivity of the $^{45}\text{Ca}^{2+}$ solution varied from 12 to 50 c.p.m./nmol.

The uptake was terminated by passing the discs quickly but gently through three 50 ml rinses of an ice-cold solution of 140 mM-NaCl/5 mM-EGTA, pH 7.4, fine forceps being used. The discs were lightly blotted on paper towels, placed in a scintillation vial containing 0.35–0.7 ml of Soluene (Packard) and dissolved by incubating them overnight at 37°C. Samples of the $^{45}\text{Ca}^{2+}$ stock solution were similarly treated with Soluene and counted to determine specific activity. The Soluene was neutralized with 20–30 μl of acetic acid before counting for radioactivity in Aquasol (New England Nuclear). Radioactivity was measured with a Packard Instrument 3255 liquid-scintillation counter, the channels-ratio system being used.

Unidirectional- Ca^{2+} -flux measurements

After removal and rinsing of the duodenum as described above, a 1.5 cm duodenal section was opened with appropriate side up on to a 2.54 cm $\times$ 2.54 cm $\times$ 0.16 cm (1 in $\times$ 1 in $\times$ $\frac{1}{16}$ in) poly-

carbonate square; it was covered with a square of identical dimensions containing a centrally bored 0.95 cm ($\frac{3}{8}$ in)-diameter hole (0.71 cm² area). The squares were clamped together tightly and fastened on two sides with rubber bands such that the centrally exposed intestinal surface was undamaged, while the crushed tissue between the squares smeared to form a seal. The tissue in its polycarbonate mount was transferred to a 50 ml beaker containing 18 ml of the prewarmed preoxygenated buffer described above; the beaker was lightly stoppered with a large cork to keep the gas line in place. After preincubation, incubation with ⁴⁵Ca²⁺ and EGTA rinsing as described above, a 0.38 cm² disc was cut out from the exposed tissue, dissolved in Soluene and counted for radioactivity. A second section of duodenum was treated identically, except for incubation under a N₂ atmosphere.

The O₂-dependent Ca²⁺ influx across the mucosal surface of mounted tissue appeared to be somewhat labile; it was important in these manipulations to be careful not to stretch or otherwise damage the villus surface and to work quickly. Ca²⁺ influx across the muscle surface of mounted tissue was always low and never observed to be O₂- or vitamin D-dependent.

Results

The time course of Ca²⁺ uptake by intestinal discs from media containing Ca²⁺ concentrations of 0.5 and 1.0 mM, is shown in Fig. 1. Clearly the discs taken from 1,25(OH)₂D₃-treated animals always take up more Ca²⁺ under an O₂ atmosphere than those from vitamin D-deficient animals at all time points. In contrast, the low Ca²⁺ uptake values obtained from discs incubated under N₂ are unaltered by vitamin treatment. Occasionally, however, when the preincubation step was omitted, discs from rats given 1,25(OH)₂D₃ showed significantly higher uptakes under N₂ than did controls under N₂.

Presumably, at each time point, the value for Ca²⁺ uptake into intestinal discs under N₂ is a good approximation of the passive diffusion of Ca²⁺ into the tissue; if in error, this approximation would probably be high because of energy supplied to the duodenal cells via anaerobic glycolysis. Uptake under O₂ should be a combination of the passive diffusion component and the O₂-dependent active transport of Ca²⁺ mediated by vitamin D that has been observed previously in intestinal mucosa (Martin & DeLuca, 1969; Schachter *et al.*, 1960; Walling & Rothman, 1969). Thus a good approximation of the O₂-dependent active transport of Ca²⁺

Fig. 1. Total and O₂-dependent Ca²⁺ uptake into duodenal segments as a function of time

In all cases the discs from 1,25(OH)₂D₃-treated rats (—), or vitamin D-deficient rats (---), were preincubated for 5 min under either O₂ or N₂ in Ca²⁺-free media before addition of 1 ml of the appropriate ⁴⁵Ca²⁺ stock solution (a and b, 0.5 mM; c and d, 1.0 mM). The O₂-N₂ differences (O₂-dependent uptake) shown in (b) and (d) were obtained from the total-uptake data in (a) and (c). Numbers of experimental animals (n) are given in parentheses; error bars show $\pm$ S.E.M. Unless given, n for vitamin D-deficient animals was the same as for 1,25(OH)₂D₃-dosed animals for the same point.

may be obtained by subtraction of the N_2 component from the O_2 component for each animal. Figs. 1(b) and 1(d) show that the O_2 -dependent uptake thus obtained is linear for about 20 min in physiological solutions containing 0.5 and 1.0 mM Ca^{2+} .

It should be made clear that much of the Ca^{2+} entering the discs is probably not sequestered in the absorptive cells themselves, but rather is trapped within the villi, submucosa, and muscle (Schachter *et al.*, 1966).

Both the mucosal and muscle surfaces of the disc are exposed to the $^{45}Ca^{2+}$ -containing media in this assay. Although previous workers have shown that the rat serosal-to-mucosal flux is passive and is unaffected by vitamin D status (Walling & Rothman, 1969; Walling *et al.*, 1974), conceivably in free-floating-disc experiments an O_2 -dependent influx into intestinal muscle might be occurring which would complicate the results. Table 1 shows that this is not the case. When the mucosal side only is exposed to the media, a substantial O_2 -dependent influx occurs that is comparable with the O_2 -dependent influx observed in free-floating discs. However, when only the muscle side is exposed, the Ca^{2+} influx into the tissue is low and is the same under an N_2 atmosphere as under an O_2 atmosphere. The absolute magnitude of the influx into muscle under N_2 is about one-half of the total influx into a free-floating disc under N_2 . These results confirm that the O_2 -dependent uptake of free-floating discs is mucosal in origin and so reflects physiological active transport of Ca^{2+} .

Fig. 2(a) shows the saturable O_2 -dependent uptake of Ca^{2+} by discs from 1,25(OH) $_2$ D $_3$ -treated and vitamin D-deficient animals as a function of Ca^{2+} concentration. The half-saturation value obtained from the x -intercept of a double-reciprocal plot of these data (Fig. 2b) is approximately the

same for vitamin-treated and vitamin D-deficient animals, being 0.8 and 0.9 mM- Ca^{2+} respectively. Under these conditions the maximum velocity of the O_2 -dependent transport (V_{max}) obtained from the y -intercept is increased about 2.5-fold by 1,25(OH) $_2$ D $_3$, from 6.7 to 16.7 nmol per cm^2 /min.

The effect of depriving the animals of food for 10–11 h before death on the O_2 -dependent Ca^{2+} influx is shown in Table 2. Of the 24 rats used in this experiment, half were deprived of food for 7½ h and then re-fed for 2–3 h, whereas the other half were deprived of food for 10–11 h. O_2 -dependent Ca^{2+} uptake into duodenal discs was then measured for both groups. As seen in Table 2, food deprivation increased the magnitude of the O_2 -dependent influx by 60–75% for vitamin D-treated and -deficient rats.

The effect of food deprivation on duodenal Ca^{2+} uptake was further examined with respect to the time course and saturability of uptake. For animals deprived of food for 9–14 h, the O_2 -dependent uptake was linear for a maximum of 15 min in tissue from 1,25(OH) $_2$ D $_3$ -treated animals; at this time the tissue appeared to be saturated with 250–300 nmol of Ca^{2+} /cm 2 when incubated in 1.0 mM- Ca^{2+} . The influx into duodenal discs under N_2 was unaffected by vitamin D status in food-deprived rats and was the same magnitude as that observed for rats fed *ad libitum* (results not shown).

Fig. 3 shows the duodenal O_2 -dependent Ca^{2+} uptake for food-deprived rats as a function of Ca^{2+} concentration. A clear saturation of the uptake occurred, with the rate of influx increased about 3-fold by treatment with 1,25(OH) $_2$ D $_3$. The approximate half-saturation values as determined from the double-reciprocal plot of these data (Fig. 3) are 1.5 mM- Ca^{2+} for food-deprived vitamin-treated animals, and 0.5 mM- Ca^{2+} for food-deprived vitamin D-deficient animals. It is not clear whether these

Table 1. Ca^{2+} influx across muscle or mucosal surfaces of intestine

Vitamin D-treated rats were dosed subcutaneously with 2.5 µg of cholecalciferol/day and used on day 3. In all cases, influx into 0.38 cm 2 discs was measured as described in the Experimental section under either 'free-floating-disc assay' or 'unidirectional flux measurements' for conditions of 1.0 mM- Ca^{2+} and 15 min incubation. The values shown are means $\pm$ S.E.M. for four, or sometimes three, animals.

Intestinal surface exposed to medium	Cholecalciferol treatment	Ca^{2+} influx into tissue (nmol/15 min per cm^2)		
		(a)	(b)	(a)–(b)
		Under O_2	Under N_2	
Both	+	204 $\pm$ 12	75 $\pm$ 6	120 $\pm$ 15
	–	127 $\pm$ 7	56 $\pm$ 3	71 $\pm$ 9
Mucosal	+	170 $\pm$ 11	38 $\pm$ 14	131 $\pm$ 4
	–	78 $\pm$ 5	39 $\pm$ 8	39 $\pm$ 4
Muscle	+	28 $\pm$ 3	26 $\pm$ 2	2 $\pm$ 4
	–	26 $\pm$ 2	32 $\pm$ 5	–6 $\pm$ 6

Fig. 2. O_2 -dependent Ca^{2+} uptake into duodenal discs from 1,25(OH) $_2$ D $_3$ -treated rats and vitamin D-deficient rats, as a function of Ca^{2+} concentration

(a) For these experiments, incubation times were within the linear region of uptake versus time; the results were then normalized to uptake/min per cm^2 . Numbers of experimental animals are given in parentheses (n), and error bars indicate $\pm$ S.E.M. Unless given, n for vitamin D-deficient animals (---) was the same as for 1,25(OH) $_2$ D $_3$ -dosed animals (—) for the same point. (b) is a double-reciprocal plot of the data shown in (a). The x-intercept indicates a half-saturation value of 0.8–0.9 mM- Ca^{2+} , whereas the maximum velocities obtained from the y-intercepts are 16.7 nmol of Ca^{2+} /min per cm^2 for segments from 1,25(OH) $_2$ D $_3$ -treated rats and 6.7 nmol of Ca^{2+} /min per cm^2 for vitamin D-deficient tissue.

Fig. 3. O_2 -dependent Ca^{2+} uptake into duodenal discs from food-deprived rats as a function of Ca^{2+} concentration

The animals were deprived of food for 10–14 h before assay; other details are as described for Fig. 2. For 1,25(OH) $_2$ D $_3$ -treated food-deprived rats, the approximate half-saturation constant for the O_2 -dependent Ca^{2+} uptake is 1.5 mM- Ca^{2+} and the $V_{\max}$ is 29 nmol of Ca^{2+} /min per cm^2 . Similar values obtained for vitamin D-deficient food-deprived rats are 0.5 mM- Ca^{2+} and 7 nmol of Ca^{2+} /min per cm^2 ; lines were drawn and intercepts calculated by using least-squares linear-regression analysis of the data.

values differ significantly from the 0.9 mM- and 0.8 mM- Ca^{2+} values obtained from fed rats. However, all the values for the half-saturation constant of the O_2 -dependent uptake, regardless of food deprivation, are near the range of values previously determined: 1.1 mM- Ca^{2+} (Papworth &

Patrick, 1970); 1.25 mM- Ca^{2+} (Walling & Rothman, 1969); and 2.0 mM- Ca^{2+} (Martin & DeLuca, 1969).

The $V_{\max}$ obtained from Fig. 3(b) for food-deprived 1,25-(OH) $_2$ D $_3$ -treated rats is 29 nmol of Ca^{2+} /min per cm^2 ; this is an increase of 74% over the value for fed animals. The $V_{\max}$ of O_2 -dependent Ca^{2+} uptake by duodenal discs from vitamin D-deficient rats is apparently unchanged by food deprivation, being 7 nmol of Ca^{2+} /min per cm^2 from

Fig. 3(b) and 6.7 nmol of Ca^{2+} /min per cm^2 from Fig. 2(b). All kinetic values were obtained by least-squares linear-regression analysis of the data.

The Ca^{2+} ionophore A23187, which has specificity for bivalent cations over univalent cations, is commonly used to increase the passive influx of Ca^{2+} into cells and vesicles (Berridge, 1975). However, when used in this system, iono-

phore A23187 did not increase any value for Ca^{2+} uptake into discs, with the exception of passive uptake under N_2 at the extremely high concentration of 52 μM -ionophore A23187. Uptake values under N_2 (in nmol of Ca^{2+} /15 min per cm^2 , were 87 ± 9 versus 66 ± 4 (32% increase, $P < 0.05$) for discs from 1,25(OH) $_2\text{D}_3$ -dosed animals, incubated with and without 52 μM -ionophore A23187 respectively, and 94 ± 4 versus 71 ± 4 (32% increase, $P < 0.005$) for discs incubated similarly from vitamin D-deficient rats.

The effect on O_2 -dependent uptake, of adding various concentrations of ionophore A23187 to the preincubation media is shown in Table 3; the Table shows results for four different concentrations of ionophore A23187 assayed under three different conditions of incubation.

At 1 μM , the ionophore significantly inhibited the O_2 -dependent uptake for discs from 1,25(OH) $_2\text{D}_3$ -dosed animals, but not for discs from vitamin D-deficient animals. However, at 8 μM , no significant effect was seen on either set of discs, suggesting a variable effect in this lower concentration range. At the two highest ionophore concentrations, a clear and significant inhibition of the O_2 -dependent component was observed. The ionophore was always added in 15–30 μl of dimethyl sulphoxide to the preincubation media. The highest concentration of dimethyl sulphoxide used, 30 μl /flask, was shown in a separate experiment to have no effect on Ca^{2+}

Table 2. *Effect of food deprivation on O_2 -dependent Ca^{2+} influx*

In this experiment, half the animals were deprived of food for 10–11 h before being killed and half were deprived of food for 7½ h, followed by 2–3 h of feeding *ad libitum*. Cholecalciferol-treated rats were dosed with 2.5 μg of cholecalciferol/day for 2 days by subcutaneous injection, and used on day 3. Conditions of assay were 1.0 mM- Ca^{2+} , 15 min incubation. $P < 0.001$ for differences between food-deprived and re-fed rats. $n = 6$ rats/group.

Treatment	O_2 -dependent Ca^{2+} uptake (nmol of Ca^{2+} /15 min per cm^2 , $\pm$ S.D.)
+Cholecalciferol	
Fed	135 ± 76
Deprived of food	221 ± 34
Vitamin D_3 -deficient	
Fed	77 ± 32
Deprived of food	44 ± 23

Table 3. *Effects of the calcium ionophore A23187 on O_2 -dependent Ca^{2+} uptake*

All rats were dosed intrajugularly, 24 h before being killed, with 50 μl of either ethanol, or ethanol containing 125 ng of 1,25(OH) $_2\text{D}_3$, as described in the Experimental section. The ionophore, in 15–30 μl of dimethyl sulphoxide, was added to the preincubation media immediately before the start of the experiment. * $P < 0.005$, † $P < 0.001$, and ‡ $P < 0.01$ when compared to the appropriate control group without ionophore A23187; abbreviation used: N.S., not significantly different.

Conditions of incubation	n	1,25(OH) $_2\text{D}_3$ treatment	[Ionophore A23187] (μM)	Uptake under O_2 – uptake under N_2 (nmol of Ca^{2+} /time (15 or 20 min))	Inhibition (%)
0.5 mM- Ca^{2+} , 20 min incubation	10	+	0	113 ± 18	—
	6	+	1	43 ± 5	62*
	10	—	0	51 ± 5	—
	6	—	1	33 ± 20	N.S.
1.0 mM- Ca^{2+} , 20 min incubation	14	+	0	164 ± 12	—
	5	+	8	133 ± 22	N.S.
	17	—	0	43 ± 8	—
	5	—	8	31 ± 10	N.S.
1.0 mM- Ca^{2+} , 15 min incubation	30	+	0	125 ± 9	—
	6	+	26	66 ± 10	47†
	6	+	~52	54 ± 23	57‡
	21	—	0	68 ± 6	—
	6	—	26	21 ± 8	69†
	6	—	~52	14 ± 5	79†

Table 4. *Effect of actinomycin D on O₂-dependent duodenal Ca²⁺ uptake*

Four groups of six rats were used with an average weight per group of about 162 ± 10 g. The rats were deprived of food for 10–12 h before being killed. At 3½ h before being killed each group of rats was dosed intrajugularly under light ether anaesthesia with one of the following: 1,25(OH)₂D₃ + actinomycin D, ethanol + actinomycin D, 1,25(OH)₂D₃ + vehicle, or ethanol + vehicle; 250 ng of 1,25(OH)₂D₃ per rat was given in 50 µl of ethanol and 2 µg of actinomycin D/g was given in a vehicle of propylene glycol/140 mM-NaCl and 10 mM-NaH₂PO₄, pH 7.4 (1:1, v/v). On killing the animals, Ca²⁺ uptake was measured for conditions of 1.0 mM-Ca²⁺, 15 min incubation. $P < 0.01$ for differences between 1,25(OH)₂D₃-treated and vitamin D-deficient rats at 3½ h without actinomycin D; $P < 0.05$ for differences between vitamin D-deficient rats with and without actinomycin D. Results are means, $\pm$ S.E.M.

Treatment	O ₂ -dependent Ca ²⁺ uptake (nmol of Ca ²⁺ /15 min per cm ² $\pm$ S.E.M.)
+ 1,25(OH) ₂ D ₃	127 $\pm$ 11
+ 1,25(OH) ₂ D ₃ + actinomycin D	25 $\pm$ 7
Vitamin D ₃ -deficient	64 $\pm$ 13
Vitamin D ₃ -deficient + actinomycin D	27 $\pm$ 7

uptake under either O₂ or N₂. At the highest concentration of ionophore, 52 µM, the incubation media was noticeably turbid on conclusion of the experiment.

Actinomycin D, an agent used to block cellular transcription, acts by penetration into intact cells where, depending on concentration, it inhibits the synthesis of ribosomal RNA, messenger RNA, and transfer RNA and secondarily inhibits protein synthesis (Scott & Tomkins, 1975). O₂-dependent intestinal Ca²⁺ uptake was blocked by 71–85% when 1.7 µg of actinomycin D/g was administered along with 1,25(OH)₂D₃ 6½ h before the assay began (results not shown). However, the inhibitor also blocked O₂-dependent Ca²⁺ uptake in vitamin D-deficient rats by 75–85% at this time point, suggesting some non-specific disruption of transport might be occurring.

Table 4 shows the result of an experiment involving actinomycin D in which the time of exposure to the inhibitor and also to 1,25(OH)₂D₃ was shortened to 3½ h. Although the tissue response to 1,25(OH)₂D₃ had not yet reached a maximum, the Ca²⁺ uptake by discs from 1,25(OH)₂D₃-dosed animals is clearly significantly increased; this uptake is blocked by 80% by simultaneous administration of actinomycin D. The O₂-dependent Ca²⁺ uptake in vitamin D-deficient animals was also inhibited, but to a lesser extent, by 58%, under these conditions. Although these results do not conclusively show that 1,25(OH)₂D₃ acts via a nuclear mechanism, they do suggest that some protein component of the active-transport system [but not necessarily a 1,25(OH)₂D₃-induced protein component] is coded for by an mRNA species with a relatively short half-life of 3–4 h.

Discussion

Although in the present method, Ca²⁺ can enter the intestinal discs from both mucosal and serosal

sides, we have shown (Table 1) that the influx across the muscle layer is low, is not O₂-dependent, and is unaffected by vitamin D status. Thus the muscle influx is contained in the N₂ component and can be subtracted, as was done by Schachter *et al.* (1960, 1961).

Depriving the rats of food for 9–14 h before assay enhances the response to 1,25(OH)₂D₃ by about 75%. A 2.5-fold increase in $V_{\max}$ due to 1,25(OH)₂D₃ treatment was observed in fed animals, whereas a 4.1-fold increase in $V_{\max}$ was calculated for food-deprived animals. Conditions of 5 min preincubation and 10–15 min incubation with 1.0 mM-Ca²⁺ are thought to be optimal for assay of the vitamin-dependent response. Under these conditions, by using food-deprived rats, an increase in intestinal Ca²⁺ uptakes of approx. 3-fold is observed in response to the hormone.

The nature of the observed enhancement by food deprivation, or inhibition by feeding, of the O₂-dependent Ca²⁺ uptake is unclear. Perhaps the tissue is saturated with unlabelled Ca²⁺ from the food, which takes time to clear, or possibly transport of other nutrients preferentially uses up ATP stores. The unlabelled Ca²⁺ from diet adhering to the intestine is probably not simply diluting the ⁴⁵Ca²⁺ in the incubation media, since the intestines are thoroughly rinsed with saline (0.9% NaCl) before use, the ratio of media volume to disc volume per flask is very large, and the duodenal discs are preincubated 5 min with agitation before addition of ⁴⁵Ca²⁺ to make a 1 mM-Ca²⁺ solution.

The results from the experiments with ionophore A23187 are difficult to interpret. If 1,25(OH)₂D₃ acts to increase entry of Ca²⁺ into intestinal cells, as has previously been suggested (Adams *et al.*, 1970; Harrison & Harrison, 1965; Rasmussen *et al.*, 1979), then ionophore A23187 would be expected at some concentration to mimic the effects of 1,25(OH)₂D₃ and increase the O₂-dependent Ca²⁺

uptake into discs from vitamin D-deficient animals. This was not the case; ionophore A23187 did not increase O_2 -dependent uptake, but either had no effect or inhibited it. The simplest explanation for the observed inhibition is that ionophore A23187 increased intracellular Ca^{2+} to toxic concentrations, either by increasing influx across the brush border, or by penetrating the absorptive cells and releasing internal Ca^{2+} stores, or both (Berridge, 1975).

Accordingly, we speculate that if $1,25(OH)_2D_3$ does act to increase the passive Ca^{2+} permeability of the absorptive-cell brush border, the hormone-dependent Ca^{2+} influx is tightly and directly coupled to intracellular packaging and extrusion. Extraneous Ca^{2+} , which enters the cells by other 'gates' such as ionophore A23187, apparently does not enter the active-transport sequence and thus might be harmful.

Reports have appeared of failure of actinomycin D to inhibit intestinal Ca^{2+} transport in rats (Tanaka *et al.*, 1971) and more recently in chicks (Bickle *et al.*, 1978). In both cases the lack of inhibition was interpreted to mean that $1,25(OH)_2D_3$ may act through a non-nuclear mechanism. However, it was not clear that the dosage schedules (substantially lower doses and longer times than were used for the data in Table 4) were sufficient to block completely intestinal mRNA production.

We have shown that $2\mu g$ of actinomycin D/g does inhibit $1,25(OH)_2D_3$ -induced intestinal Ca^{2+} transport by 80% when administered simultaneously with $1,25(OH)_2D_3$ $3\frac{1}{2}$ h before assay. However, no firm conclusions can be drawn from this experiment concerning a nuclear or non-nuclear mechanism for $1,25(OH)_2D_3$, since a similar inhibition was observed in tissue from vitamin D-deficient animals. Although straightforward precedents exist for elucidation of a steroid-hormone mechanism with actinomycin D (e.g. DeAngelo & Gorski, 1970), results with $1,25(OH)_2D_3$ may well continue to be ambiguous until clearly defined rapidly appearing molecular products are found, or another mechanism is elucidated. Our actinomycin D results do argue that the induction of RNA *de novo* and of protein synthesis by $1,25(OH)_2D_3$ cannot be ruled out as a medium for stimulating Ca^{2+} transport, as Bickle, *et al.* (1978) have claimed.

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