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# Characterization of 1,25-Dihydroxyvitamin $D_3$-Dependent Calcium Uptake in Isolated Chick Duodenal Cells

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**Summary.** The *in vivo* and *in vitro* effects of 1,25-dihydroxyvitamin $D_3$ (1,25-$(OH)_2D_3$) on calcium uptake by isolated chick duodenal cells were studied. *In vivo*, 1,25-$(OH)_2D_3$ given orally to vitamin D-deficient chicks increased the initial rate of calcium uptake by cells prepared 1 hr after administration of the hormone. The rate was stimulated approximately 100%, 17 to 24 hr after repletion. *In vitro*, pre-incubation of 1,25-$(OH)_2D_3$ with cells from D-deficient chicks increased the cellular rate of calcium uptake in a concentration-dependent relationship. Enhancement was found with $10^{-15}$ M, was maximal at $10^{-13}$ M, and was diminished at higher ($10^{-11}$ M) concentrations. Stimulation was observed after a pre-incubation period as brief as 1 hr. The potency order for vitamin $D_3$ analogs was 1,25-$(OH)_2D_3$ = 1-$(OH)D_3$ > 25-$(OH)D_3$ > 1,24,25-$(OH)_3D_3$ > 24,25-$(OH)_2D_3$ > $D_3$. The maximal enhancement in calcium uptake induced by the analogs was the same, only the concentration at which the cell responded was different. The effectiveness of 1,25-$(OH)_2D_3$ was five orders of magnitude greater than $D_3$. Kinetically, 1,25-$(OH)_2D_3$ increased the $V_{max}$ of calcium uptake; the affinity for calcium ($K_m$ = 0.54 mM) was unchanged. The enhanced uptake found after the cells were pre-incubated for 2 hr with the hormone was completely blocked by inhibitors of protein synthesis. 1,25-$(OH)_2D_3$, *in vitro*, also increased calcium uptake in cells isolated from D-replete chicks. The maximal rates of uptake were the same in cells from D-deficient and D-replete animals. The hormone had no effect on calcium efflux from cells. Calcium uptake in microvillar brush-border membrane vesicles was increased by 1,25-$(OH)_2D_3$. These findings suggest that the *in vitro* cell system described in this paper represents an appropriate model to examine the temporal relationships between 1,25-$(OH)_2D_3$ induction of calcium transport and specific biochemical correlates.

**Key Words** vitamin D-dependent calcium uptake · intestinal cell calcium transport · intestinal brush-border calcium uptake · vitamin D-deficient chicks · vitamin D, mechanism of action · 1,25-dihydroxycholecalciferol

## Introduction

Evidence has accumulated indicating that the biologically active form of vitamin D 1,25-$(OH)_2D_3$ functions, at least in part, as a steroid hormone regulating intestinal transport of calcium (for reviews, *see* Norman, 1980; Stanbury, 1980; DeLuca, 1983). However, the precise mechanism by which the hormone controls the handling of calcium is still not clear. Some of the difficulties in resolving the biochemical action of 1,25-$(OH)_2D_3$ stem from the complexities inherent in studies in which the steroid is administered to an animal, *in vivo*, or added, *in vitro*, to relatively intact intestinal loops and segments. *In vitro* studies utilizing the embryonic chick duodenum in organ culture have contributed much to the understanding of the mechanism of action of the vitamin D metabolite (Corradino & Wasserman, 1971; Corradino, 1973; Franceschi & DeLuca, 1981*a*,*b*; Bishop et al., 1983). In addition, it has been reported that intestinal cells isolated from D-deficient rats injected *in vivo* with 1,25-$(OH)_2D_3$ have increased calcium uptake (Bronner et al., 1983). It has also been noted that cells derived from rat duodenal segments treated *in vitro* with a relatively high concentration of 1,25-$(OH)_2D_3$, but not with 25-$(OH)D_3$, show enhanced calcium uptake (Freund & Bronner, 1975). Associated with this increase in calcium uptake is the induction of a calcium binding protein. However, the cell system was not examined further. In this paper, we use duodenal cells isolated from D-deficient chicks to demonstrate rapid responses to vitamin D metabolites. The cells have an increased calcium uptake when the bird is injected *in vivo* and when the cells are pre-incubated *in vitro* with very low physiological concentrations of 1,25-$(OH)_2D_3$. Moreover, the characteristics of this cell system as a model for investigating how the steroid hormone enhances intestinal calcium uptake are described. A preliminary account of part of this work has been presented in abstract form (Liang et al., 1984).

## Materials and Methods

### Animals

One-day-old white leghorn cockerels were kept in a darkened room for 3 weeks and maintained on a vitamin D-deficient diet (Teklab test diet TD 75007), containing 0.37% Ca and 0.37% P, as standardized by Matsumoto et al. (1980). The chicks had free access to deionized water. Vitamin D deficiency was monitored by serum Ca, 6.9 ± 0.2 and 8.6 ± 0.3 mg/dl, in D-deficient and -repleted birds, respectively (Liang et al., 1982*a*). To replete, the chicks were given orally 1 μg of 1,25-$(OH)_2D_3$, dissolved in 200 μl of propylene glycol.

### Isolation of Cells

Duodenum cells were isolated by a modification of the method described by Kimmich (1970). The preparative procedure was carried out at room temperature. The isolation medium was 240 mM mannitol, 3 mM $K_2PO_4$, 1 mM $MgCl_2$, 5 mM NaCl, 0.5 mM β-hydroxybutyrate, 2.5 mM glutamine, 10 mM mannose, 1 mg/ml bovine serum albumin, and 20 mM HEPES, adjusted to pH 7.4 with KOH. After sacrifice of the chick, the duodenum was excised and trimmed of adhering pancreas. The luminal contents were flushed out with 5 ml of isolation medium. The intestine was slit lengthwise and washed again with medium. The tissue was then cut into segments about 2 cm in length and placed in a 50-ml polyethylene beaker containing 20 ml of medium. Hyaluronidase, at a final concentration of 1 mg/ml of medium, was added and the mixture incubated in a Blue M Constant Temperature bath at 37°C for 30 min with vigorous shaking (130 oscillations/min, 2 cm path length). At the end of the incubation, the cells were detached by gentle agitation of the tissue segments with a glass rod. The suspension was filtered through two layers of cheese cloth and the cells in the filtrate sedimented by centrifugation at 120 × *g* for 2 min in a 15-ml polyethylene tube. The cells were washed three times by resuspension in 15 ml of isolation medium with a plastic pipette followed by recentrifugation. The washed cells were finally resuspended at a concentration of 1 to 2 mg of cell protein/ml of isolation medium. Microscopic examination of the preparations revealed single cells and some cell aggregates, a few of these containing as many as 20 cells.

### Pre-incubation of Cells with 1,25-$(OH)_2D_3$

To 2 ml of a suspension of isolated cells in a 50-ml polyethylene centrifuge tube, 10 μl of 1,25-$(OH)_2D_3$ or other vitamin D metabolites, dissolved in ethanol, or ethanol alone was added. Unless indicated otherwise, the pre-incubation period was for 2 hr at 37°C, with vigorous shaking (130 oscillations/min, 2 cm path length). This constant agitation was sufficient to maintain an oxygen-rich medium, as was evident from the findings that the ATP content and other functions of the cell did not change during the 2-hr pre-incubation period (*see* the Table, below). The pre-incubation medium was then diluted with 6 ml of a washing solution containing (in mM): 140 KCl, 0.5 β-hydroxylbutyrate, 2.5 glutamine, 10 mannose, and 10 HEPES, adjusted to pH 7.4 with KOH, and the cells sedimented by centrifugation at 450 × *g* for 3 min. Cells were resuspended in the washing medium to a concentration of 3 to 6 mg of cell protein/ml for determination of $Ca^{2+}$ uptake.

### Uptake of Calcium

A 100-μl aliquot of the cell suspension was pre-equilibrated in a shaking water bath for 5 min at 37°C. Uptake was initiated by the addition of 100 μl of a solution containing 140 mM KCl, 10 mM HEPES, adjusted to pH 7.4 with KOH, and 2 mM $^{45}CaCl_2$ (0.5 μCi). Except when noted, the final $Ca^{2+}$ concentration was 1 mM and the uptake period was 5 min. The uptake was terminated by removing 180 μl of the reaction mixture and adding it to an Eppendorf centrifuge tube containing 1 ml of ice-cold stopping solution consisting of 140 mM KCl, 10 mM HEPES, and 2 mM EGTA, adjusted to pH 7.4. The cells were centrifuged for 2 min, and the pellet washed with 1 ml of stopping solution and recentrifuged. The washing step was repeated again. To the cell pellet, 0.5 ml of 1 N NaOH was added, and the mixture let stand for at least 30 min. The mostly solubilized cells were transferred to a scintillation vial and combined with 0.5 ml of water used to rinse the Eppendorf tube. Radioactivity was measured in a scintillation counter. Uptake of $Ca^{2+}$ was linear with respect to cell protein, within the range used in the present experiments (0.2 to 0.8 mg protein), and with respect to time of uptake, for at least 5 min.

### Efflux of Calcium

Cells isolated from vitamin D-deficient chicks were pre-incubated with 1,25-$(OH)_2D_3$ or ethanol (control) for 2 hr. The cells were incubated with 1 mM $^{45}CaCl_2$ for 5 min, as described for the uptake studies. To examine efflux, 0.18 ml of the cell suspension was pipetted into an Eppendorf centrifuge tube containing 1.2 ml of a solution containing 140 mM KCl, 2 mM EGTA, and 10 mM HEPES, pH 7.4. The temperature was 25°C. At different times, the cells were centrifuged and the $^{45}Ca^{2+}$ remaining in the cells determined as described above. To determine the zero-time $^{45}Ca^{2+}$ efflux, the cells, after taking up $^{45}Ca^{2+}$, were pipetted into ice-cold efflux medium and immediately centrifuged.

### Brush-Border Membrane Vesicles

Duodenal brush-border membrane vesicles were obtained by the procedure described by Schultz et al. (1982). Briefly, the crude brush borders were prepared by the $Ca^{2+}$ precipitation technique. The microvillar membranes were dissociated from the crude brush borders by homogenization in 0.5 M Tris/HCl, pH 7.2. The microvillar membranes were separated from the core fraction on a discontinuous 37 to 60% (wt/wt) glycerol gradient. The final brush-border membrane preparations, essentially all right-side out (Shultz et al., 1982), were enriched 16- to 20-fold in alkaline phosphatase specific activity relative to the mucosal homogenate.

To measure $Ca^{2+}$ uptake by the brush-border membrane vesicles, the vesicles were pre-equilibrated with a buffer solution containing 140 mM choline chloride and 10 mM HEPES, adjusted to pH 7.4. To initiate uptake, 25 μl of a reaction medium containing 140 mM KCl, 10 mM HEPES, pH 7.4, and 2 mM $^{45}CaCl_2$ (about 10 μCi/ml) was added to 25 μl of the membrane-vesicle suspen-

sion, and the reaction incubated at 20°C in a shaking water bath. Uptake was terminated by the addition of ice-cold stopping solution, consisting of 140 mM KCl, 2 mM EGTA, and 10 mM HEPES, adjusted to pH 7.4, and the suspension applied on a Millipore filter (DAWP 02500). The filter was rapidly washed twice with 3 ml of stopping solution. The labeled $Ca^{2+}$ retained on the filter was estimated by liquid scintillation spectrometry.

### Metabolism and Integrity of the Isolated Cells

The ATP content of cells freshly prepared and of cells after preincubation with 1,25-$(OH)_2D_3$ was measured. Cells were sedimented by brief centrifugation in a table top clinical centrifuge and then resuspended in 1.5 ml of the buffered KCl solution. To 1.2 ml of the cell suspension 80 $\mu$l of 60% perchloric acid was added. The mixture was kept for 20 min in an ice-bath and the precipitate removed by centrifugation. An aliquot of the supernatant was titrated to neutrality with KOH (1.2 ml extract plus 138 $\mu$l of 5 M KOH and 72 $\mu$l of 500 mM HEPES buffer). After centrifugation, ATP was determined as described by Kornberg (1955*a*).

To measure the incorporation of [$^3$H] amino acids into protein, the cells were washed and suspended in 2 ml of the KCl buffer. To 0.9 ml of the cell suspension 0.1 ml of a [$^3$H] amino acids solution (100 $\mu$l of the amino acid mixture, 75 $\mu$Ci, in 2.4 ml McCoy's 5a medium) was added. After a 30-min incubation, the cells were washed with the KCl buffer twice to remove extracellular labeled amino acids. The cells were resuspended in KCl buffer and perchloric acid was added to the suspension to a final concentration of 3%. The precipitated protein was sedimented by centrifugation, washed, redissolved in 1 N NaOH, and radioactivity counted.

Cellular integrity was assessed by measuring the leakage of lactic dehydrogenase activity. Cells suspended in isolation medium were incubated for 30 min at 37°C with vigorous shaking in the absence and presence of 0.3% Triton X-100. After incubation the cells were centrifuged. Dehydrogenase activity in the supernatant was determined (Kornberg, 1955*b*).

The metabolism of the cells was also evaluated by measuring the rate of production of $^{14}CO_2$ from [1-$^{14}$C] glucose. A 0.5-ml suspension of cells in isolation medium was mixed with 0.5 ml of 4 mM labeled glucose (0.1 $\mu$Ci) in the same medium. A hanging well containing 0.2 ml of hyamine hydroxide was inserted in the serum stopper used to cap the tube. The reaction proceeded at 37°C with vigorous shaking. After the incubation, 0.1 ml of 2 N $H_2SO_4$ was injected into the reaction mixture. The reaction tube was placed in a shaking water bath at 37°C for an additional hour to allow for complete trapping of the $^{14}CO_2$ by the hydroxide. The rate of $^{14}CO_2$ production was linear with time for at least 1 hr.

### Materials

Hyaluronidase (bovine tests, type IS, 300 U/mg), anisomycin, cycloheximide and cholecalciferol were purchased from Sigma Chemical Co. [$^3$H] amino acids, [1-$^{14}$C] glucose, and $^{45}Ca^{2+}$ were from New England Nuclear. 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$, and 1,24,25-$(OH)_3D_3$ were generously provided by Dr. M. Uskokovic of Hoffman-LaRoche. 1-(OH)$D_3$ and 25-(OH)$D_3$ were gifts of the Upjohn Co. Hyamine hydroxide was obtained from Amersham Searle.

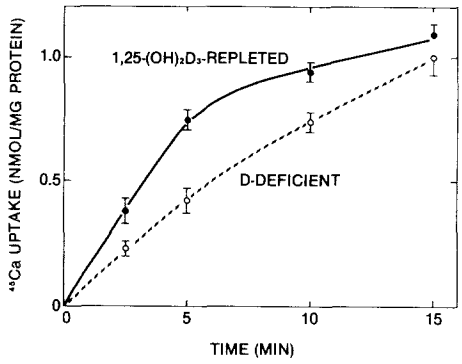


**Fig. 1.** $Ca^{2+}$ uptake in duodenal cells isolated from vitamin D-deficient chicks and from birds repleted with 1,25-$(OH)_2D_3$ 17 hr before sacrifice. Each datum represents the mean ± SE for six experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

## Results

### $Ca^{2+}$ Uptake in Cells from D-Deficient Chicks and from Animals Repleted *in Vivo*

Figure 1 shows the time course of uptake of 1 mM $^{45}Ca^{2+}$ in duodenal cells isolated from vitamin D-deficient cockerals and from birds given 1 $\mu$g of 1,25-$(OH)_2D_3$ 17 hr before sacrifice. With cells from both kinds of chicks, accumulation of $^{45}Ca^{2+}$ was linear with time of incubation for about 5 min. This finding suggested that during this initial period of incubation influx of $Ca^{2+}$ into the cell predominated. The rate of accumulation then slowed. Presumably, at this phase of the incubation the rate of $Ca^{2+}$ influx was being approached by the rates of active $Ca^{2+}$ efflux and passive leak. The experiment also demonstrated that repletion with the hormone increased the initial (5 min) rate of $^{45}Ca^{2+}$ uptake about 80%, from 0.42 ± 0.05 nmol/mg of cell protein · 5 min in cells from D-deficient animals to 0.75 ± 0.04 in cells from D-repleted chicks. When approximate steady states were attained, at about 15 min of incubation, the amounts of $^{45}Ca^{2+}$ taken up by cells from D-deficient and D-repleted animals were not significantly different.

### Time Course of the Cellular Responses to the Repletion of D-Deficient Chicks

To determine the time at which the cell uptake of $Ca^{2+}$ was enhanced after the D-deficient chicks

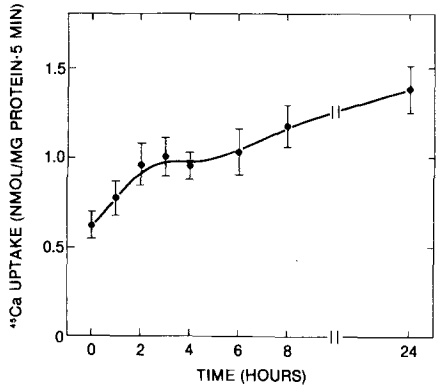


**Fig. 2.** Time course of the cellular response to a single injection of 1,25-$(OH)_2D_3$ given to D-deficient chicks. Values represent the mean ± SE for six experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

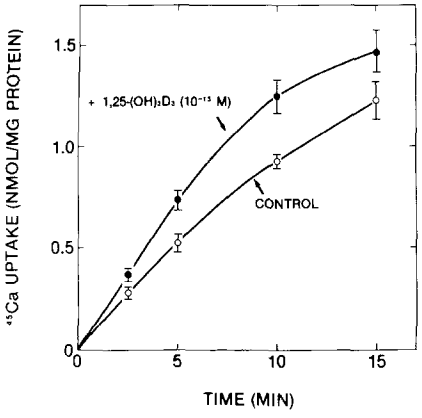


**Fig. 3.** $Ca^{2+}$ uptake in cells following the pre-incubation of the cells *in vitro* with 1,25-$(OH)_2D_3$ or ethanol alone (control) for 2 hr. Each datum represents the mean ± SE for three experiments, each from different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

were repleted, cells were isolated at different periods of time after the single oral injection of 1 μg 1,25-$(OH)_2D_3$. As shown in Fig. 2, cells prepared 1 hr after administration had an increased $Ca^{2+}$ uptake, almost 30%. This rapid enhancement became highly significant by the second hour, 55%, from 0.62 ± 0.07 to 0.96 ± 0.12 nmol/mg of cell protein 5 min ($P < 0.025$). The rate of uptake did not seem to increase further for several hours thereafter. Then, after the sixth hour, a second phase of stimulation became evident. This latter enhancement was more gradual and by 24 hr after repletion, $Ca^{2+}$ uptake was 1.38 ± 0.12 nmol/mg of cell protein × 5 min, an increase greater than 100% the rate in cells from D-deficient animals.

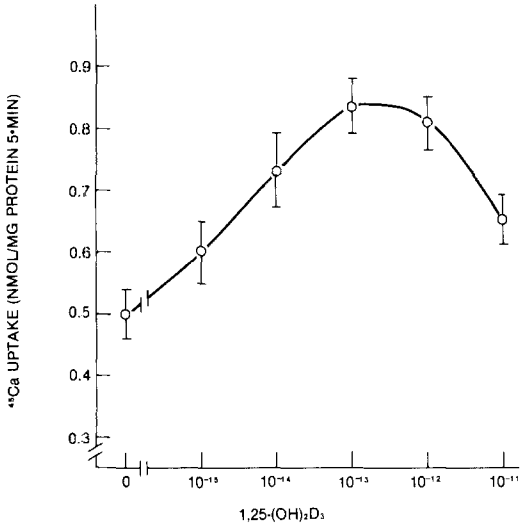


**Fig. 4.** Effect of the concentration of 1,25-$(OH)_2D_3$ pre-incubated *in vitro* for 2 hr with cells from D-deficient chicks on initial rate of $Ca^{2+}$ uptake. Each datum represents the mean ± SE for 12 to 18 experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

## $Ca^{2+}$ Uptake in Cells Pre-incubated *in Vitro* with 1,25-$(OH)_2D_3$

Cells from D-deficient chicks were pre-incubated *in vitro* with 1,25-$(OH)_2D_3$ for 2 hr. Then, the rate of $Ca^{2+}$ uptake was determined. As described in Fig. 3, rates of $Ca^{2+}$ uptake in cells pre-incubated for 2 hr with ethanol alone (control) and with 1,25-$(OH)_2D_3$ ($10^{-13}$ M) were linear with time for 5 min. $Ca^{2+}$ uptakes in cells pretreated with other concentrations of 1,25-$(OH)_2D_3$ as well as with various concentrations of different vitamin $D_3$ metabolites were also linear with time for at least 5 min (*data not illustrated*). In addition, the data in Fig. 3 demonstrate that pre-incubation of the cells *in vitro* with 1,25-$(OH)_2D_3$ significantly enhanced the cellular uptake of $Ca^{2+}$.

Figure 4 shows how pre-incubation with different physiological concentrations of 1,25-$(OH)_2D_3$ affected $Ca^{2+}$ uptake. A concentration as low as $10^{-15}$ M 1,25-$(OH)_2D_3$ increased the uptake of $Ca^{2+}$ by 20%, from 0.50 ± 0.04 to 0.60 ± 0.05 nmol/mg of cell protein × 5 min ($P < 0.005$). Maximum uptake was found with $10^{-13}$ M hormone; in this experiment a value of 0.83 ± 0.04 nmol/mg of cell protein × 5 min was attained, representing an enhancement of 66% ($P < 0.001$). The dose-response relationship was biphasic, for with higher concentrations of steroid ($10^{-11}$ M) uptake was diminished from the maximal value.

**Table.** Effects of the two-hour pre-incubation period with 1,25-$(OH)_2D_3$ on the integrity and metabolism of the isolated intestinal cells[a]

| Experimental condition | ATP content | Release of LDH into medium | | | Amino acid incorporation into protein | Glucose metabolism |
|---|---|---|---|---|---|---|
| | | − Triton | + Triton | Ratio (+ : −) | | |
| Freshly prepared cells | 5.8 ± 0.3 | 0.24 ± 0.01 | 1.31 ± 0.06 | 5.5 ± 0.4 | 1070 ± 49 | — |
| After 2 hr of pre-incubation with: | | | | | | |
| Control | 5.4 ± 0.5 | 0.27 ± 0.02 | 1.72 ± 0.07 | 6.4 ± 0.3 | 1131 ± 221 | 29.8 ± 2.7 |
| 1,25-$(OH)_2D_3$ ($10^{-13}$ M) | 5.6 ± 0.5 | 0.28 ± 0.04 | 1.78 ± 0.13 | 6.7 ± 0.9 | 1231 ± 236 | 30.7 ± 3.3 |
| 1,25-$(OH)_2D_3$ ($10^{-11}$ M) | — | 0.24 ± 0.03 | 1.56 ± 0.03 | 5.9 ± 0.4 | 1077 ± 166 | 31.2 ± 2.2 |

[a] Duodenum cells were isolated from D-deficient chicks and measurements made immediately thereafter. Other aliquots of the cell suspensions were incubated for 2 hr with either ethanol (control) or the indicated concentration of 1,25-$(OH)_2D_3$. Units of ATP content are nmol · $mg^{-1}$ protein. Units of lactic dehydrogenase (LDH) activity are $\mu$mol NAD · $mg^{-1}$ protein · $min^{-1}$. Units of [$^3$H] amino-acid incorporation into perchloric acid precipitable protein are cpm · $mg^{-1}$ protein · 30 $min^{-1}$. Units of glucose metabolism are nmol $^{14}CO_2$ · $mg^{-1}$ protein · $hr^{-1}$. Values represent the mean ± SE of 9, 6, 4, and 6 experiments for determination of cellular ATP content, release of LDH, amino-acid incorporation into protein, and glucose metabolism, respectively.

Because the increase in $Ca^{2+}$ uptake found in cells pre-incubated for 2 hr with 1,25-$(OH)_2D_3$ might reflect an enhanced metabolic state of the cells (Schachter & Rosen, 1959; Kendrick et al., 1981) and/or a sustained integrity of the cells rather than a direct effect on transport, it was important to assess the viability of the cells before and after pre-incubation with the hormone. These questions were addressed by a variety of measurements, described in the Table. In one test, that of cellular ATP content, it was found that freshly prepared duodenum cells had an ATP content of 5.8 ± 0.3 nmol/mg cell protein. The ATP content of the cells did not significantly change after the cells were pre-incubated for 2 hr with $10^{-13}$ M 1,25-$(OH)_2D_3$ or with the vehicle control, these values being 5.6 ± 0.5 and 5.4 ± 0.5, respectively. In another test, the ability of the cells to incorporate amino acids into protein was evaluated. The data in the Table show that the rates of incorporation of labeled amino acids into perchloric acid precipitable protein did not differ between cells, assayed immediately after isolation, and cells, pre-incubated for 2 hr with the hormone and with ethanol. In a third test, there were no differences in the rates of formation of [$^{14}$C]$CO_2$ from 1-[$^{14}$C] glucose between newly prepared cells and cells after the pre-incubation period. These results indicated that the general metabolism of the cells was not measurably altered by the 2-hr pre-incubation. The viability of the cells was further examined by measuring the leakage of the cytosolic enzyme, lactic dehydrogenase, before and after the pre-incubation. Enzyme activity released into the medium in the presence and absence of Triton X-100 was used to assess plasma membrane integrity. With freshly prepared cells, the dehydrogenase activity found in the extracellular medium after detergent treatment increased more than 5 times (Table), indicating a highly preserved plasma membrane. The ratio of released dehydrogenase activity in the presence of Triton relative to the activity in the absence of detergent did not decrease after the cells were pre-incubated for 2 hr with 1,25-$(OH)_2D_3$ or with the vehicle control. Thus, these experiments also supported the view that the viability of the cells was not compromised after the 2-hr period of pre-incubation prior to $Ca^{2+}$ uptake. Further, the fall in the rate of $Ca^{2+}$ uptake found with the relatively "high" concentration of $10^{-11}$ M 1,25-$(OH)_2D_3$ (Fig. 4) was not due to a "toxic" effect of the hormone, at least as detectable by leak of a cytosolic enzyme, amino acid incorporation into protein, and glucose metabolism (Table).

Stimulation of $Ca^{2+}$ uptake could be observed with an *in vitro* pre-incubation period as brief as 1 hr (Fig. 5). As shown, when cells from D-deficient animals were pre-treated with $10^{-13}$ M 1,25-$(OH)_2D_3$ for 60 min, $Ca^{2+}$ uptake was increased about 25%, from 0.58 ± 0.05 in control cells to 0.72 ± 0.05 nmol/mg of cell protein in cells exposed to the hormone ($P < 0.02$). When the pre-incubation period was 2 hr, the enhancement was 60% ($P < 0.005$). The magnitude

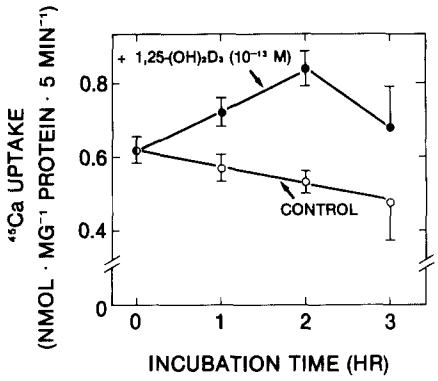


**Fig. 5.** Effect of the duration of the period in which cells were pre-incubated with 1,25-$(OH)_2D_3$ on the initial (5 min) rate of $Ca^{2+}$ uptake. Each datum represents the mean ± SE for six to eight experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

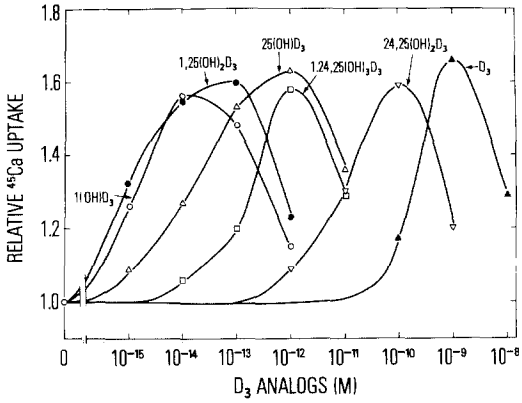


**Fig. 6.** Specificity of vitamin $D_3$ analogs acting *in vitro* in stimulating $Ca^{2+}$ uptake in cells from D-deficient chicks. Values are the averages of at least five experiments, each carried out in quadruplicate. For clarity of illustration, standard errors of each datum are not shown. These are approximately 10% of the illustrated mean values

of the percentage increase over the control was the resultant of both an absolute increase in $Ca^{2+}$ uptake in the 1,25-$(OH)_2D_3$-treated cells coupled to a slight decrease in uptake by the control cells. Prolongation of the *in vitro* pretreatment period to 3 hr resulted in an initial rate of $Ca^{2+}$ uptake less than that found with a 2-hr pre-incubation. To be noted, however, was the further decrease in $Ca^{2+}$ uptake in the control cells with the prolonged pre-incubation. It was not unreasonable to postulate that cellular integrity might have declined by the third hour of pre-incubation in a saline medium at 37°C with constant agitation.

### Specificity of Vitamin $D_3$ Analogs in Stimulating $Ca^{2+}$ Uptake

Duodenal cells isolated from vitamin D-deficient chicks were pre-incubated *in vitro* for 2 hr with different concentrations of several vitamin $D_3$ analogs. As shown in Fig. 6, a maximal stimulation of approximately 60% was found for all the compounds tested. Moreover, for every analog, the effect was biphasic, in that the rate of $Ca^{2+}$ uptake increased with increasing analog concentration until a maximal response was obtained, and then uptake diminished with higher concentrations of the steroid. The effectiveness of 1-$(OH)D_3$ in stimulating $Ca^{2+}$ uptake approximated that of 1,25-$(OH)_2D_3$. The potency order for the analogs was 1,25-$(OH)_2D_3$ = 1-$(OH)D_3$ > 25-$(OH)D_3$ > 1,24,25-$(OH)_3D_3$ > 24,25-$(OH)_2D_3$ > $D_3$. The effectiveness of 1,25-$(OH)_2D_3$ was approximately 5 orders of magnitude greater than that for $D_3$. Since chick intestine was known to be capable of converting 1-$(OH)D_3$ to 1,25-$(OH)_2D_3$ (Holick et al., 1976), the possibility that the efficacy of 1-$(OH)D_3$ was due in part to its conversion to 1,25-$(OH)_2D_3$ during the 2-hr pre-incubation period was not precluded.

### Effect of 1,25-$(OH)_2D_3$ on the Kinetics of $Ca^{2+}$ Uptake

Figure 7 describes the kinetics of $Ca^{2+}$ uptake by cells from D-deficient animals and by cells pre-incubated *in vitro* with $10^{-13}$ M 1,25-$(OH)_2D_3$ for 2 hr. With cells from D-deficient birds the rate of $Ca^{2+}$ uptake increased as the $Ca^{2+}$ concentration increased. The hormone stimulated uptake at each of the concentrations tested. The relationship between rate of uptake and $Ca^{2+}$ concentration obeyed Michaelis-Menten kinetics (Fig. 7, inset). An apparent $K_m$ of 0.54 mM $Ca^{2+}$ was calculated and this value was not altered after treatment of the cells with 1,25-$(OH)_2D_3$. The $V_{max}$, however, was increased after pre-incubation with the hormone, from 1.05 to 1.60 nmol/mg of cell protein · 5 min.

### Inhibitors of Protein Synthesis Block the 1,25-$(OH)_2D_3$-Dependent $Ca^{2+}$ Uptake

Accumulated evidence from several studies suggested that at least some of the responses of the intestine to 1,25-$(OH)_2D_3$ were mediated by a nuclear mechanism (Haussler et al., 1968; Chen & DeLuca, 1973; Franceschi & DeLuca, 1981*b*). Should this be the case, then there should be a requirement for *de novo* protein synthesis. Therefore, we tested whether inhibitors of protein synthesis in chick

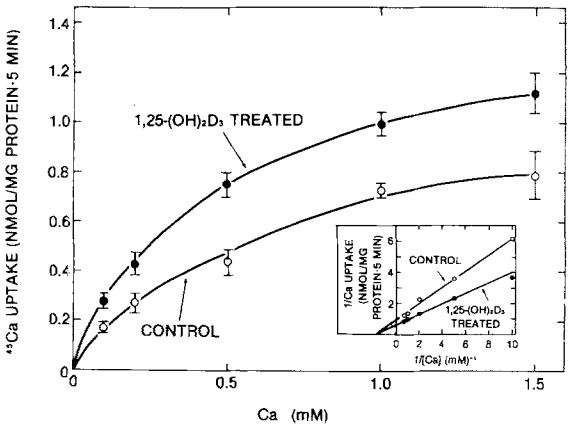


**Fig. 7.** Effect of 1,25-$(OH)_2D_3$ on the kinetics of $Ca^{2+}$ uptake in cells pre-incubated with the hormone *in vitro*. The indicated $Ca^{2+}$ concentrations, from 0.1 to 1.5 mM, reflect the final concentration of $CaCl_2$ added to the uptake medium. Inset is a Lineweaver-Burk transformation of the data. Each value represents the mean ± SE for five experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

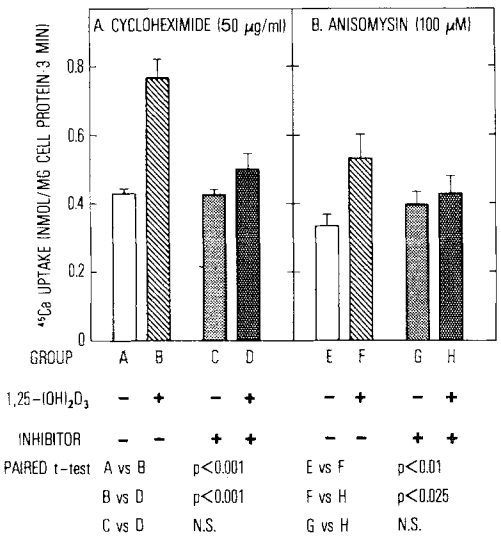


**Fig. 8.** Effect of inhibitors of protein synthesis on the 1,25-$(OH)_2D_3$-induced increase in $Ca^{2+}$ uptake in isolated duodenum cells. Inhibitors were added to the cell suspension 20 min before the addition of the steroid hormone. Values are the mean ± SE for six experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

preparations (Franceschi & DeLuca 1981*b*; Liang et al., 1982*b*) altered the increase in $Ca^{2+}$ uptake induced in cells pre-incubated *in vitro* for 2 hr with physiological concentrations of the steroid. Figure 8 illustrates the effect of the translational inhibitor cycloheximide on basal $Ca^{2+}$ uptake and on the uptake dependent on the action of $10^{-13}$ M 1,25-$(OH)_2D_3$. Cycloheximide (50 μg/ml) did not alter the basal rate of $Ca^{2+}$ uptake (Fig. 8, *A* versus *C*). The 1,25-$(OH)_2$-$D_3$-dependent uptake, *i.e.*, the difference between Fig. 8*B* and *A*, was nearly completely blocked by the inhibitor of protein synthesis (*B* versus *D*). Similar results were obtained with anisomycin, an inhibitor of polypeptide chain elongation (Fig. 8).

## Effect of 1,25-$(OH)_2D_3$, *in Vitro*, on Cells from Vitamin D-Replete Chicks

Cells were isolated from D-deficient chicks and from animals given 1 μg of 1,25-$(OH)_2D_3$ 17 hr previously. The two kinds of cells were pre-incubated with different concentrations of 1,25-$(OH)_2D_3$ for 2 hr and then initial (5 min) rates of $Ca^{2+}$ uptake determined. As shown in Fig. 9, in the absence of hormone in the pre-incubation mixture, *i.e.*, the vehicle control, uptake was almost 50% greater in cells from D-repleted birds than in cells from D-deficient chicks, consistent with the findings shown in Fig. 1. $Ca^{2+}$ uptakes in cells from D-repleted chicks were further enhanced by treatment *in vitro*

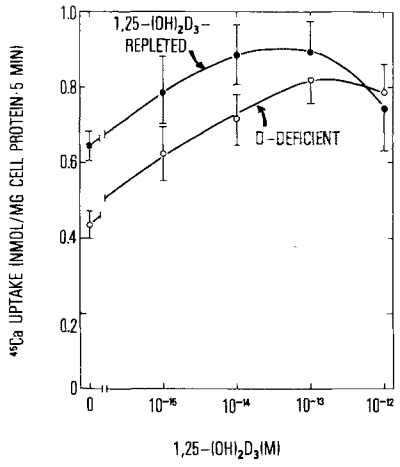


**Fig. 9.** Effect of 1,25-$(OH)_2D_3$, pre-incubated *in vitro*, on cells from vitamin D-repleted and -deficient chicks. Each datum represents the mean ± SE for five experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

with 1,25-$(OH)_2D_3$. For example, with $10^{-13}$ M, 1,25-$(OH)_2D_3$ uptake was increased from 0.64 ± 0.04 to 0.89 ± 0.08 nmol/mg of cell protein, a 39% increase ($P < 0.01$). At the same concentration of steroid in the pre-incubation mixture, $Ca^{2+}$ uptake in cells from D-deficient cockerels was raised from 0.43 ± 0.04 to 0.82 ± 0.07 nmol/mg of cell protein, a

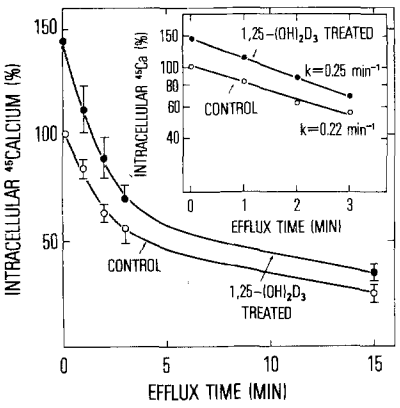


**Fig. 10.** Effect of 1,25-$(OH)_2D_3$ on $Ca^{2+}$ efflux from cells. Inset shows first-order plots of the data. Experimental details are reported in the text. Values represent the mean ± SE of six experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

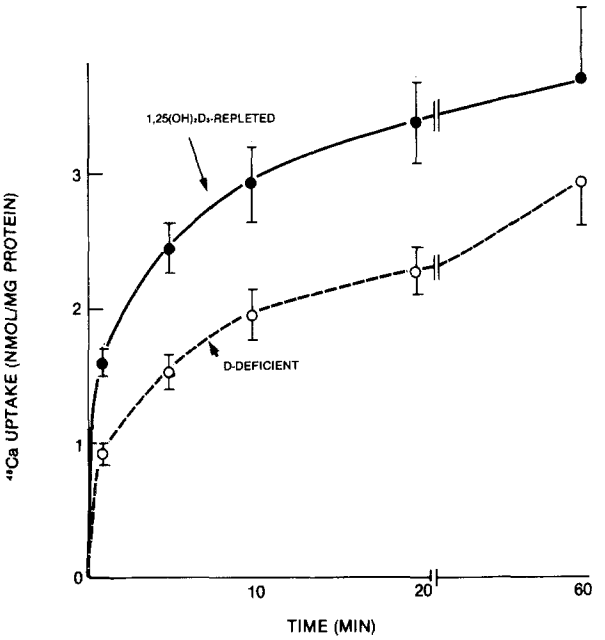


**Fig. 11.** $Ca^{2+}$ uptake into duodenal brush-border membrane vesicles from D-deficient chicks and animals repleted with 1,25-$(OH)_2D_3$, as described in the text. Each datum represents the mean ± SE for four experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

89% increase ($P < 0.01$). Yet, the fully stimulated rates of $Ca^{2+}$ uptake in the two cell types were not significantly different, 0.89 ± 0.08 *vs.* 0.82 ± 0.07 nmol/mg of cell protein. Further, the dose-response curves were similar, except for the lower percentage stimulation in cells from D-repleted animals. Thus, cells from both D-deficient and D-repleted birds were stimulatable by the *in vitro* exposure to 1,25-$(OH)_2D_3$ and the maximal rate of $Ca^{2+}$ uptake was the same in the two types of cells.

## Effect of 1,25-$(OH)_2D_3$ on $Ca^{2+}$ Efflux from Cells

Because of the report that 1,25-$(OH)_2D_3$ might regulate $Ca^{2+}$ pump activity at the baso-lateral membrane of rat duodenal cells (Ghijsen & Van Os, 1982), we examined the effects of the hormone on $Ca^{2+}$ efflux in chick cells treated *in vitro* with 1,25-$(OH)_2D_3$. In this experiment (Fig. 10), cells from D-deficient animals were pre-incubated with $10^{-13}$ M, 1,25-$(OH)_2D_3$ or ethanol for 2 hr, followed by the uptake of $Ca^{2+}$ during a 5-min incubation. Then, efflux was initiated by dilution into an EGTA-containing medium, as described in Materials and Methods. Efflux from treated and control cells exhibited first-order kinetics. Calculation of the efflux rate constant (Fig. 10, inset) revealed no significant difference between cells pre-incubated with and without 1,25-$(OH)_2D_3$, the $k$ values being 0.25 and 0.22 $min^{-1}$, respectively. This result indicated no difference in the rates of $Ca^{2+}$ efflux in $Ca^{2+}$-loaded cells between chick cells pre-incubated or not treated with 1,25-$(OH)_2D_3$. Further, this finding ruled out the possibility that a 1,25-$(OH)_2D_3$-induced decreased in $Ca^{2+}$ efflux was the mechanism by which the hormone increased $Ca^{2+}$ uptake (Fig. 3), as was also negated by the observation that the initial rate of $Ca^{2+}$ uptake was enhanced by 1,25-$(OH)_2D_3$ (Fig. 3). In another experiment in which the rates of $Ca^{2+}$ efflux from cells pre-incubated with $10^{-13}$ and $10^{-11}$ M 1,25-$(OH)_2D_3$ were compared, the efflux rate constants were identical (*data not illustrated*). This result also precluded the possibility that the higher concentration of 1,25-$(OH)_2D_3$ ($10^{-11}$ M) decreased the rate of $Ca^{2+}$ accumulation relative to the rate found with $10^{-13}$ M (Fig. 6) because of a stimulated rate of efflux.

## $Ca^{2+}$ Uptake in Brush-Border Membrane Vesicles

To demonstrate additionally that 1,25-$(OH)_2D_3$ increased the activity of the $Ca^{2+}$ transport system in the plasma membrane of the enterocyte, brush-border membrane vesicles were prepared from D-deficient chicks and from birds administered with 1 μg 1,25-$(OH)_2D_3$ 17 hr previously. Figure 11 shows that the uptake of $Ca^{2+}$ into membrane vesicles from the D-repleted chick was 73% greater than the uptake into membrane vesicles from control animals, 1.59

± 0.11 and 0.92 ± 0.07 nmol/mg of membrane protein · min, respectively. Uptake was significantly increased in vesicles from 1,25-$(OH)_2D_3$-treated chicks at all points on the time-course of uptake, except at 1 hr when steady-state uptakes presumably were approached.

To resolve the question as to whether the 1,25-$(OH)_2D_3$-dependent $Ca^{2+}$ uptake by brush-border membrane vesicles represented the increased transport of the divalent cation into the membrane vesicles or its enhanced binding, the effect of osmolarity of the medium on uptake was determined. Intravesicular space was decreased by increasing the medium osmolarity from 0.3 to 0.7 osM. As shown in Fig. 12, uptakes of $Ca^{2+}$ at 1 min were found to be inversely proportional to medium osmolarity suggesting uptakes into osmotically active membrane vesicles; thus, transport across the brush-border membrane in vesicles from both D-deficient and 1,25-$(OH)_2D_3$-repleted chicks. When extrapolated to infinite medium osmolarity, no significant difference in binding was found between vesicles from D-deficient and repleted animals. Therefore, the 1,25-$(OH)_2D_3$-dependent increase in $Ca^{2+}$ uptake represented the hormone-induced stimulation of $Ca^{2+}$ transport across the luminal segment of the duodenal cell plasma membrane, rather than an increase in $Ca^{2+}$ binding to the brush-border membrane.

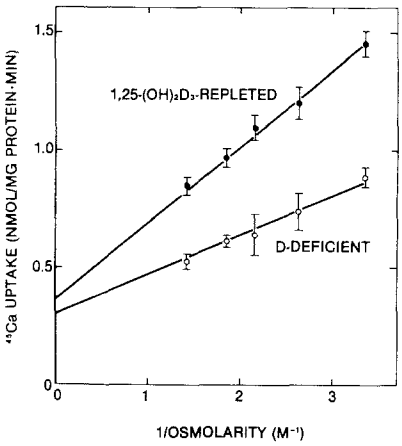


**Fig. 12.** Effects of osmolarity on the $Ca^{2+}$ uptake into brush-border membrane vesicles prepared from D-deficient and 1,25-$(OH)_2D_3$-repleted chicks. Each datum represents the mean ± SE of three experiments, each with different cell preparations from different animals. In each experiment, uptakes were carried out in quadruplicate

## Discussion

The present results demonstrated that $Ca^{2+}$ uptake in duodenal cells isolated from the chick was increased by 1,25-$(OH)_2D_3$. The response was observed when the hormone was administered to the animal, *in vivo,* in agreement with an earlier report with intestinal cells from the rat (Bronner et al., 1983). Further, we now found that $Ca^{2+}$ uptake was enhanced after the cells were pre-incubated with 1,25-$(OH)_2D_3$, *in vitro.* The hormone-induced increased accumulation of labeled $Ca^{2+}$ may be attributed to changes in several cell properties. One possibility is that 1,25-$(OH)_2D_3$ increased the binding of $Ca^{2+}$ to the external surface of the plasma membrane rather than stimulating the uptake into an intracellular compartment. This alternative seems unlikely because uptakes were terminated by the addition of EGTA to the incubation medium, and afterwards, the cells were washed rapidly but thoroughly with the chelator. This stopping procedure should have removed readily accessible externally bound $Ca^{2+}$. A second possibility is that the steroid hormone decreased the efflux of $Ca^{2+}$ from the cell. However, direct measure of $Ca^{2+}$ efflux from control and 1,25-$(OH)_2D_3$-treated cells revealed that the efflux rates were not significantly different (Fig. 10). A third possibility, and the one that we currently support, is that 1,25-$(OH)_2D_3$ increased the influx of $Ca^{2+}$ into the cell. Accumulations of $Ca^{2+}$ by control and hormone-treated cells were linear with time during the uptake period indicating that the influx phase of $Ca^{2+}$ uptake into the cell predominated. Further, the hormone was found to increase the uptake of $Ca^{2+}$ into brush-border membrane vesicles, indicating stimulation in the transport of the divalent cation across the luminal segment of the enterocyte plasma membrane (Fig. 11). The present results, however, do not preclude the possibility that 1,25-$(OH)_2D_3$ might have also increased the uptake of $Ca^{2+}$ across the basolateral segment in the isolated intact duodenal cell. This latter possibility appears unlikely, however, because 1,25-$(OH)_2D_3$ was reported not to mediate alterations in the serosal-to-mucosal flux of $Ca^{2+}$ (Adams & Norman, 1970; Nemere et al., 1984). The present findings do not address the question whether 1,25-$(OH)_2D_3$ altered the intracellular calcium buffering capacity of the cells by affecting the uptake of $Ca^{2+}$ by intracellular organelles, such as mitochondria (Bikle et al., 1980) and Golgi membranes (MacLaughlin et al., 1980). However, after the 2-hr preincubation period with 1,25-$(OH)_2D_3$ the cells from vitamin D-deficient chicks had an increased uptake of $Ca^{2+}$ but calcium binding protein could not be detected by immunoassay.[1]

[1] C.T. Liang, J. Barnes, and B. Sacktor, *unpublished observations.*

Cells from D-deficient chicks were exquisitely sensitive to 1,25-$(OH)_2D_3$. A concentration as low as $10^{-15}$ M stimulated $Ca^{2+}$ uptake by 20%, after cells were pre-incubated with the hormone *in vitro* for 2 hr. Half-maximal stimulation was obtained with $10^{-14}$ M steroid. Maximal uptake, representing an increase of approximately 60%, was found with $10^{-13}$ M. Thus, the response of the isolated cell to the hormone was several orders of magnitude greater than that reported for the cultured embryonic chick duodenum (Franceschi & DeLuca, 1981*a*). Concentrations of 1,25-$(OH)_2D_3$ higher than $10^{-13}$ M resulted in uptakes less than the maximal value. The explanation for this biphasic dose-response relationship is not known. However, the possibility that the higher concentrations of 1,25-$(OH)_2D_3$ decreased $Ca^{2+}$ accumulation by increasing the rate of $Ca^{2+}$ efflux was ruled out. In addition, it was found that the general metabolism and integrity of the cells were not adversely affected by the higher concentration of 1,25-$(OH)_2D_3$ (Table). Interestingly, similar biphasic concentration-response relationships were reported previously for the actions of 1,25-$(OH)_2D_3$ in modulating the exchange of $Ca^{2+}$ in cultured bone cells (Eilam et al., 1980) and the incorporation of sulfate into cultured chondrocytes (Corvol et al., 1978). It should be noted that in the present experiments 1,25-$(OH)_2D_3$ and other vitamin $D_3$ metabolites were added in ethanol to the pre-incubation media. Serum was never present, as chick serum was known to contain a vitamin D metabolite binding protein (Corradino, 1978). Indeed, when 1,25-$(OH)_2D_3$ was added in serum, from vitamin D-deficient chicks, to the medium and the cells pre-incubated with the hormone for 2 hr, the 1,25-$(OH)_2D_3$ concentration-$Ca^{2+}$ uptake curve was shifted to the right.[1] With serum present, a 15% stimulation of $Ca^{2+}$ uptake was seen with $10^{-13}$ M, half-maximal stimulation was found with $10^{-12}$ M, and a maximal 56% enhancement in uptake was obtained with $10^{-10}$ M 1,25-$(OH)_2D_3$. However, the concentration of the vitamin D binding protein in chick serum is 4 $\mu$M and its association constant for 1,25-$(OH)_2D_3$ is about $10^9$ $M^{-1}$ (Bouillon et al., 1980). Thus, the calculated free concentrations of 1,25-$(OH)_2D_3$ will be between $10^{-13}$ and $10^{-14}$ M, the range found here to regulate $Ca^{2+}$ uptake in the isolated cell system (Fig. 4).

The ability of several vitamin $D_3$ analogs to stimulate $Ca^{2+}$ uptake in isolated chick duodenal cells generally parallel their ability to increase intestinal $Ca^{2+}$ absorption *in vivo,* to enhance uptake in cultured embryonic duodenal cells *in vitro,* and to induce intestinal $Ca^{2+}$ binding protein (Corradino, 1978; Franceschi & DeLuca, 1981*a*). In the present experiments the order of potency was 1,25-$(OH)_2D_3$ = 1-$(OH)D_3$ > 25$(OH)D_3$ > 1,24,25-$(OH)_3D_3$ > 24,25-$(OH)_2D_3$ > $D_3$, with the effectiveness of 1,25-$(OH)_2D_3$ being approximately 5 orders of magnitude greater than that of $D_3$. It should be noted, however, that the maximal enhancement in $Ca^{2+}$ uptake achieved by each of the analogs was the same, approximately 60%; only the concentration at which the cell responded and the apex attained was different. Although the precise relationship between the hormone-induced increase in $Ca^{2+}$ transport and the induction of $Ca^{2+}$-binding protein remains to be clarified, we found that 1-$(OH)D_3$ and 1,25-$(OH)_2D_3$ were equipotent in stimulating $Ca^{2+}$ uptake in the isolated cells and the two analogs were equipotent in inducing the $Ca^{2+}$-binding protein (Corradino, 1978). In contrast, 1-$(OH)D_3$ was only 18% as active as 1,25-$(OH)_2D_3$ in stimulating $Ca^{2+}$ uptake in the organ cultured system (Franeschi & DeLuca, 1981*a*). Whether this apparent discrepancy was due to differences in experimental protocol is not clear.

Examination of the effect of the pre-incubation of cells with 1,25-$(OH)_2D_3$ on the kinetics of cell $Ca^{2+}$ uptake indicated that the hormonal treatment increased the $V_{max}$ of uptake with no change in the $K_m$ for $Ca^{2+}$ (Fig. 7). Similar findings were described for $Ca^{2+}$ uptake in the cultured embryonic chick duodenal cell (Franceschi & DeLuca, 1981*a*) and for unidirectional $Ca^{2+}$ flux in ileal segments of vitamin D-deficient and calciferol-treated chicks (Wong & Norman, 1975). The present estimate of the $K_m$ for $Ca^{2+}$, 0.54 mM, was in close agreement with the value of 0.45 mM, reported in the earlier study (Franceschi & DeLuca, 1981*a*).

Significant increases in cell uptake of $Ca^{2+}$ were found 1 to 2 hr after the injection of 1,25-$(OH)_2D_3$ into D-deficient chicks (Fig. 2) and 1 hr after preincubating isolated cells with the steroid hormone (Fig. 5). Earlier time periods were not tested because a statistically significant enhancement appreciably less than that seen at 1 hr (25 to 30%) would be difficult to discern, considering the error inherent in the present technique. Nevertheless, this rapid response time was quicker than the 4.5 to 6 hr needed to detect increases in $Ca^{2+}$ uptake in organ cultures of chick duodenum incubated with 1,25-$(OH)_2D_3$ (Franceschi & DeLuca, 1981*a*), and the 4 hr required to see significant increases in *in situ* ligated duodenal loops after the intracardiac administration of the hormone to D-deficient chicks (Wasserman et al., 1982). In partially or fully vitamin D-repleted chicks, however, the response to 1,25-$(OH)_2D_3$ was faster, 1 hr (Wasserman et al., 1982), and it could be as rapid as 14 min (Nemere et al., 1984).

In addition to the distinction between the duodenal cell isolate and other preparations in the time

at which the response to 1,25-$(OH)_2D_3$ was first detected, the time course of the response might vary with different intestinal systems. In the present study, *in vivo* repletion of the D-deficient chick with 1,25-$(OH)_2D_3$ induced a rapid enhancement in $Ca^{2+}$ uptake in the first 1 to 2 hr after injection, followed by a slower increase in uptake which remained elevated for 24 hr post-repletion (Fig. 2). In a previous study, enhancement of $Ca^{2+}$ absorption by duodenal loops *in situ* was first found 6 hr after the administration of 1,25-$(OH)_2D_3$ to rachitic chicks, uptake reached a plateau at 24 hr and then decreased after 48 hr (Moriuchi & DeLuca, 1976). In another report, intestinal absorption of $Ca^{2+}$ in D-deficient chicks *in vivo* was increased after 4 hr, reached a peak at 18 hr and then declined rapidly (Spencer et al., 1978). In the same study, however, $Ca^{2+}$ transport measured *in vitro* in everted intestinal sacs was significantly enhanced by 2 hr, with maximal stimulation at 8 hr. With a different intestinal preparation, $Ca^{2+}$ uptake by brush-border membrane vesicles was increased at 2 hr after repletion; uptake reached a maximal level of 8 hr, and this high activity was maintained for 24 hr (Fontaine et al., 1981). Similar findings were obtained in another study with brush-border membrane vesicles but, in addition, it was found that the stimulation of $Ca^{2+}$ uptake by the membrane vesicle preparation preceded the enhancement of $Ca^{2+}$ transport by the *in situ* duodenal loop (Bikle et al., 1983). With cultured embryonic chick duodenal cells, significant increases in $Ca^{2+}$ uptake could be detected at 4.5 to 6 hr with the response continuing to increase for up to 24 hr (Franceschi & DeLuca, 1981*a*). These comparisons suggest that, in general, except for the time at which the response was initially detected, there appeared to be a relatively consistent pattern in the time course of the response in chicks, although specific differences in the time course were evident with different intestinal preparations. It should be noted that in the present study the contents of calcium and phosphate in the diet were relatively low (0.37% for each), but identical to those used in earlier studies (Matsumoto et al., 1980). Whether the difference between the diet used here and in other studies bear on the sensitivities of the various preparations to 1,25-$(OH)_2D_3$ has yet to be examined.

Because in some experiments increases in $Ca^{2+}$ absorption appeared too rapidly to be explained by the genome activation mechanism of 1,25-$(OH)_2D_3$ action and induction of $Ca^{2+}$-binding protein, and the injection of antinomycin D and cycloheximide into rachitic chicks failed to block the ability of 1,25-$(OH)_2D_3$ to stimulate $Ca^{2+}$ transport as measured in isolated duodenal loops *in vivo* (Bikle et al., 1978), it was proposed that 1,25-$(OH)_2D_3$ influenced two distinct phases of $Ca^{2+}$ absorption, *i.e.*, an initial transport response (mediated by an unknown mechanism or involving a change in phospholipid metabolism in the brush-border membrane (Fontaine et al., 1981)), and a second response dependent on *de novo* protein synthesis (Wasserman et al., 1982; Nemere et al., 1984). On the other hand, in the embryonic chick duodenal organ culture, a protein comigrating during two-dimensional electrophoresis with chick $Ca^{2+}$-binding protein was induced by 1,25-$(OH)_2D_3$ at least 2 hr before the $Ca^{2+}$ uptake response (Bishop et al., 1983). In the present study, the increased $Ca^{2+}$ uptake measured 2 hr after exposing the isolated duodenal cells to 1,25-$(OH)_2D_3$ was found to be completely blocked by the *de novo* protein synthesis inhibitors cycloheximide and anisomycin. Thus, the *in vitro* cell system described in this paper represents an appropriate model to examine the temporal relationship between 1,25-$(OH)_2D_3$ induction of $Ca^{2+}$ transport and specific biochemical correlates. Such studies are currently under way in our laboratory.

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