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Decreased intestinal calcium absorption *in vivo* and normal brush border membrane vesicle calcium uptake in cortisol-treated chickens: Evidence for dissociation of calcium absorption from brush border vesicle uptake

(vitamin D/microvilli/transport)

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ABSTRACT **The influence of cortisol on intestinal calcium transport was studied in isolated duodenal loops and brush border membrane (BBM) vesicles of vitamin D-deficient or replete chickens. Four- to five-week-old vitamin D-deficient cockerels were dosed intraperitoneally with 1 μg of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] alone 15 hr before sacrifice or in combination with 1, 3, or 5 mg of cortisol 24 and 48 hr before sacrifice. After a 1-μg dose of 1,25-$(OH)_2D_3$ the *in situ* intestinal ligated loop technique revealed a 60% increase in calcium absorption compared to control birds ($P \leq 0.001$). However, the administration of cortisol in various doses (3 and 5 mg) to chickens given 1,25-$(OH)_2D_3$ resulted in significant decreases in intestinal calcium transport *in vivo* ($P \leq 0.05$; $P \leq 0.05$). When intestinal BBM vesicles were prepared from birds treated in a manner identical with that described above, there was no observable difference between calcium uptake in BBM vesicles of the 1,25-$(OH)_2D_3$-treated birds and that of the cortisol plus 1,25-$(OH)_2D_3$-treated birds. 1,25-$(OH)_2D_3$-treated and 1,25-$(OH)_2D_3$ plus cortisol-treated chicks had intestinal BBM vesicle uptakes that were significantly greater than those of vitamin D-deficient controls ($P \leq 0.02$; $P \leq 0.025$). These data show that *in vivo* intestinal calcium transport may be markedly reduced in the presence of normal intestinal BBM vesicle calcium uptake. This suggests that factors other than BBM calcium uptake (e.g., protein synthesis or contraluminal membrane events) play an important role in the movement of calcium from the intestinal lumen into the bloodstream and extracellular fluid of the organism.**

Vitamin D_3 via its active metabolite, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], is responsible for the active transport of calcium in the proximal intestine (1–3). Much has been learned in recent years of the metabolism of vitamin D, but the precise cellular and molecular scheme of events leading to increased intestinal calcium transport remains unknown. It is known, for example, that 1,25-$(OH)_2D_3$ is rapidly localized to the nucleus of intestinal cells (4–8). In all likelihood, 1,25-$(OH)_2D_3$ enters the cell and associates with a cytosolic binding protein (9, 10) prior to translocation into the nucleus (11). In the nucleus it increases RNA polymerase II activity (11), RNA synthesis (12), chromatin template activity (13), and the synthesis of mRNA for calcium-binding protein (14). Although it is known that intestinal calcium-binding protein is a vitamin D-dependent protein (14, 15), its exact role in the translocation of calcium is not agreed upon. However, it is likely that 1,25-$(OH)_2D_3$ functions via events involving alteration in the genome. Recently, controversy has arisen concerning the necessity of protein synthesis in calcium transport after a dose of 1,25-$(OH)_2D_3$. Bikle *et al.* (16) reported that calcium transport was not altered in the presence of protein synthesis or RNA polymerase inhibitors. Recently, Rasmussen *et al.* (17) and Matsumoto *et al.* (18) reported that cycloheximide pretreatment decreased alkaline phosphatase content but did not block the effect of 1,25-$(OH)_2D_3$ treatment on calcium or phosphate transport in brush border membrane (BBM) vesicles. Rasmussen and coworkers (17–20) have shown that 1,25-$(OH)_2D_3$ alters the ratios of phosphatidylcholine to phosphatidylethanolamine in BBM, and it has been stated that changes in the fluidity of the membranes may be associated with changes in calcium uptake. These investigators have taken this as evidence that BBM events are critical in the transport of calcium and phosphorus across the intestinal cell; further, on the basis of their work and the experiments of Bikle *et al.*, Rasmussen and his group have suggested that protein synthesis may not be important in the mechanism of action of 1,25-$(OH)_2D_3$. Franceschi and DeLuca (21), using cultured embryonic duodenum, have recently shown that calcium uptake by intestinal cells is abolished in the absence of protein synthesis. This would suggest that protein synthesis is indeed vital in the movement of calcium across the cell. The evidence available to date is conflicting as to which event induced by 1,25-$(OH)_2D_3$ in the intestinal cell is central to the movement of calcium across the cell. In order to determine if BBM events are critical in transcellular calcium transport, we determined the effects of cortisol treatment on *in vivo* intestinal calcium transport and *in vitro* BBM vesicle calcium uptake. We observed the dissociation of BBM vesicle calcium uptake and *in vivo* calcium transport. Normal BBM vesicle calcium uptake was observed in the presence of depressed *in vivo* calcium transport. The significance of these findings is discussed below.

MATERIALS AND METHODS

General. All solvents were spectral or high-performance liquid chromatography grade or were distilled prior to use. Ultraviolet spectra of 1,25-$(OH)_2D_3$ were taken in absolute ethanol with a Beckman model 35 recording spectrophotometer. Radioactivity was determined with a Beckman LS-9000 β-scintillation counter and a Searle model 1185 γ counter.

Materials. Tris, mannitol, Tris adenosine 5′-triphosphate, 2,6-dichloroindophenol, phenolphthalein glucuronide, glucose oxidase reagent, glucose 6-phosphate, maleic acid, sodium cacodylate, uridine 5′-diphospho-D-galactose, *N*-acetylglucosamine, and cortisol acetate were obtained from Sigma. Glycerol

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Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; BBM, brush border membrane; KMT, KCl/mannitol/Tris.

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was purchased from Eastman Kodak, and ultrapure sucrose (density gradient grade) was purchased from Schwarz/Mann. Tween 80 was purchased from Baker. $^{45}CaCl_2$ and [U-^{14}C]glucose were obtained from New England Nuclear. $^{47}CaCl_2$ and uridine diphospho-D-[6-^{3}H]galactose were purchased from Oak Ridge National Laboratory (Oak Ridge, TN) and Amersham, respectively. Nonradiolabeled 1,25-$(OH)_2D_3$ was obtained from Hoffmann–La Roche.

Animals and Dosing Procedures. One-day-old White Leghorn cockerels were obtained from the Silver Lake Hatchery and Breeding Farms (Silver Lake, MN). They were fed a soy-protein, vitamin D-deficient, 1% calcium, and 0.5% phosphorus diet (22) ad lib for 4–5 weeks. The chicks were dosed intraperitoneally with 1 μg of 1,25-$(OH)_2D_3$ in 0.2 ml of ethanol alone 15 hr before sacrifice or in combination with various doses of cortisol (1, 3, or 5 mg per chick) in 0.2 ml of isotonic saline containing 3% Tween 80 at 24 and 48 hr before experimentation. Control birds received vehicle alone.

Preparation of Brush Border Membrane Vesicles. Nonfasted cockerels were killed by cervical dislocation and the duodenal loops were removed and rinsed with ice-cold 0.9% NaCl. All subsequent steps were performed either on ice or at 4°C. Thereafter, the mucosa was scraped and BBM vesicles were prepared by a procedure modified from Schmitz *et al.* (23). The mucosal scrapings were homogenized in a Waring blender for 15 s at 4°C in 20 vol (wt/vol) of 50 mM mannitol/2 mM Tris·HCl, pH 7.1, and the homogenate was filtered through a piece of nylon mesh of 0.5 mm pore size. Calcium chloride was then added to the filtrate to a final concentration of 10 mM. After standing in an ice bath with occasional stirring for 10 min, the suspension was centrifuged in a Beckman SS-34 rotor for 20 min at 7,500 × *g*. The pellet was discarded and the supernatant was subjected to a second centrifugation at 20,000 × *g* for 20 min. The resulting pellet containing the crude brush border fragments was resuspended in 10 ml each of double-distilled water and 1.4 M Tris·HCl (pH 7.1) to obtain a final Tris concentration of 0.7 M. This suspension was homogenized with a motor-driven Teflon pestle (5 up and down strokes). Tris was used in order to disrupt brush border fragments into microvillus membrane and core material (24). Discontinuous 37–60% (wt/wt) glycerol gradients (50 ml) were prepared in polyallomer tubes by layering 10 ml each of 60%, 45%, 42%, 40%, and 37% glycerol which contained 50 mM $MgCl_2$. The Tris/pellet mixtures (6.5 ml per gradient) were layered onto the top of each gradient. Gradients were placed on an SW 25.2 rotor and centrifuged at 91,000 × *g* for 15 min at 4°C in a Beckman LS-50 ultracentrifuge. One clearly visible band was recovered, washed in 2–3 times its volume of double-distilled water, and centrifuged at 40,000 × *g* for 20 min. This preparation contained nearly pure BBM vesicles as assessed by electron microscopy and membrane markers.

Protein was determined according to Bradford (25), using crystalline bovine serum albumin (fraction V) as a standard. Ribonucleic acid was used as a marker for ribosomal contamination and was determined after extraction (26) with orcinol reagent by the method of Meijbaum (27). Deoxyribonucleic acid was determined by the diphenylamine method of Burton (28) and served as a marker for nuclei.

Enzyme Assays. Mg^{2+}-stimulated ATPase (EC 3.6.1.3) and Na^+,K^+-stimulated ATPase (EC 3.6.1.3) were determined by the method of Fujita *et al.* (29). The amount of P_i released was determined by the method of Chen *et al.* (30). Na^+,K^+-ATPase activity was calculated as the difference between the observed activity in the presence of Na^+, K^+, and Mg^{2+} and Mg^{2+}-ATPase activity. This enzyme was measured as a marker for basolateral membranes. Sucrase (EC 3.2.1.26) was determined as described by Messer and Dahlquist (31) and was used as a marker for brush border membranes. Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Earl and Korner (32), and β-glucuronidase (EC 3.2.1.31) was assayed according to Gianetto and DeDuve (33). These enzymes were used as markers for mitochondrial and lysosomal contamination, respectively. Glucose-6-phosphatase (EC 3.1.3.9) and galactosyltransferase (EC 2.4.1.38) activities were measured as described (34, 35) and were used as marker enzymes for endoplasmic reticulum and Golgi membranes, respectively.

Electron Microscopy. Brush border membrane preparations were initially fixed in Trump's universal fixative (36) containing 1% glutaraldehyde and 4% (vol/vol) formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. After three rinses in 0.1 M phosphate buffer, the membranes were postfixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.2. The membranes were then rinsed three times in distilled water, stained en bloc in 2% (wt/vol) uranyl acetate (60°C) for 30 min, dehydrated by successive ethanol (60–100%, vol/vol) washing, and infiltrated with and embedded in Spurr's low-viscosity embedding resin (37). Thin sections were cut with a diamond knife on an LKB Ultratome III, poststained with 0.3% lead citrate, and examined with a Philips 300 transmission electron microscope.

***In Situ* Duodenal Loop Experiments.** Duodenal calcium ^{47}Ca absorption was assessed by a modification of an *in situ* ligated loop technique as described by Omdahl *et al.* (22). Animals were operated upon while they were under light ether anesthesia. The peritoneal cavity was opened and a 10-cm portion of the duodenal loop was exposed. The distal end of the loop was ligated and a 19-gauge needle was inserted into the proximal end, through which 0.2 ml of an isotonic saline solution containing 5 μCi (1 Ci = 3.7×10^{10} becquerels) of ^{47}Ca and 10 mM $CaCl_2$ was injected. The loop was then ligated. The duodenum was replaced in the peritoneal cavity and the incision was closed with wound clips. After 30 min the animals were killed, the duodenal loops (10 cm) were removed, and radioactivity remaining in the loops was determined. The absorption of ^{47}Ca was expressed as a percentage of the total dose removed from the loop.

Glucose Uptake in BBM Vesicles. Glucose uptake by BBM vesicles was measured by a filtration technique. The membrane vesicles were preincubated at 25°C in 50 mM KCl/50 mM mannitol/2 mM Tris·HCl, pH 7.4 (KMT), and 2 mM D-glucose containing 10^6 cpm/ml of [U-^{14}C]glucose for 20 min. At time zero, KMT-buffered NaCl was added to bring the final D-glucose and NaCl concentrations to 1 mM and 100 mM, respectively. At appropriate times, aliquots (100 μg of protein) were removed, rapidly collected on Millipore filters (HAWP 02500), and washed with 10 ml of ice-cold KMT buffer containing 0.2 mM phloretin. Retained [^{14}C]glucose was determined by liquid scintillation counting.

Ca^{2+} Uptake in BBM Vesicles. Calcium uptake by BBM vesicles was measured by a membrane filtration technique as described by Rasmussen *et al.* (17) with minor modifications. The membrane vesicles were suspended in KMT buffer (pH 7.4) and preincubated for 20 min at 25°C. Calcium uptake was initiated by addition of BBM vesicles to KMT buffer, which contained in final concentration 1.25 mM $CaCl_2$, ^{45}Ca at 10^7 cpm/ml, and membrane protein at approximately 1 mg/ml (25°C). Membrane protein (100 μl: 90 ± 3.2 μg, mean ± SEM, n = 24) was removed from the incubation at appropriate time intervals and pipetted directly onto Millipore filters (HAWP 02500) previously soaked overnight in 10 mM $CaCl_2$. The filters were then quickly rinsed with 10 ml of KMT buffer containing 20 μM $LaCl_3$. Retained ^{45}Ca was determined by liquid scintillation counting.

Statistical Analysis. Where appropriate, statistical comparisons of independent sample means were made by using Student's unpaired *t* test at the 95% level of confidence (two-tailed).

RESULTS

Electron microscopy revealed that the BBM fraction was composed of homogeneous membranous material of vesicular conformation (Fig. 1) that contained no contaminating organelles as assessed by morphological ultrastructure examination. The amount of electron-dense core material contained within the intravesicular space in our preparations was light to moderate, and was similar to that found by Schmitz *et al.* (23). In addition, the membrane-enclosed vesicles are covered by glycocalyx, indicating that the vesicles are right side out.

Purity of the BBM was assessed by measurement of various specifically localized enzymes. On the basis of the brush border sucrase activity, a 20-fold purification relative to the original homogenate was observed. The preparations contained 5% of the original homogenate brush border sucrase activity. Very small amounts of RNA were detected in the vesicles (0.03% of that in the original homogenate), whereas succinate dehydrogenase, β-glucuronidase, galactosyltransferase, and DNA were undetectable. The vesicles contained only a trace of glucose-6-phosphatase and approximately 0.6% of the Na^+,K^+-ATPase present in the homogenate, representing an increase in Na^+, K^+-ATPase activity approximately 1/10th that of the enrichment in sucrase. Isolation of BBM vesicles was routinely complete within 5 hr so that uptake experiments could be carried out the same day.

Brush border membranes from vitamin D-deficient chicks exhibited the sodium-dependent glucose uptake described by Hopfer *et al.* (38) as a characteristic of intestinal BBM vesicles. The pattern of uptake obtained in the presence of 100 mM NaCl was similar to that reported by Hopfer *et al.* (38), Murer and Hopfer (39), and Rasmussen *et al.* (17) for vesicles prepared from rat and chicken intestine.

In order to determine the dosage of $1,25\text{-}(OH)_2D_3$ and cortisol required to effect *in vivo* duodenal calcium absorption, a series of experiments were performed. Measurements of calcium absorption in vitamin D-deficient chicks administered $1,25\text{-}(OH)_2D_3$ alone or in combination with various doses of cortisol are shown in Fig. 2. As expected, calcium absorption was significantly increased in the $1,25\text{-}(OH)_2D_3$-treated birds, compared to the vitamin D-deficient birds ($P \leq 0.001$); however, a significant inhibition (30–32%) of the vitamin D_3-treated chicks' calcium absorption was observed with 3 and 5 mg ($P \leq 0.05$; $P \leq 0.05$) but not 1 mg of cortisol when given 24 and 48 hr before observation.

In BBM vesicle preparations of both vitamin D-deficient and replete chickens, an initial rapid uptake of calcium occurred

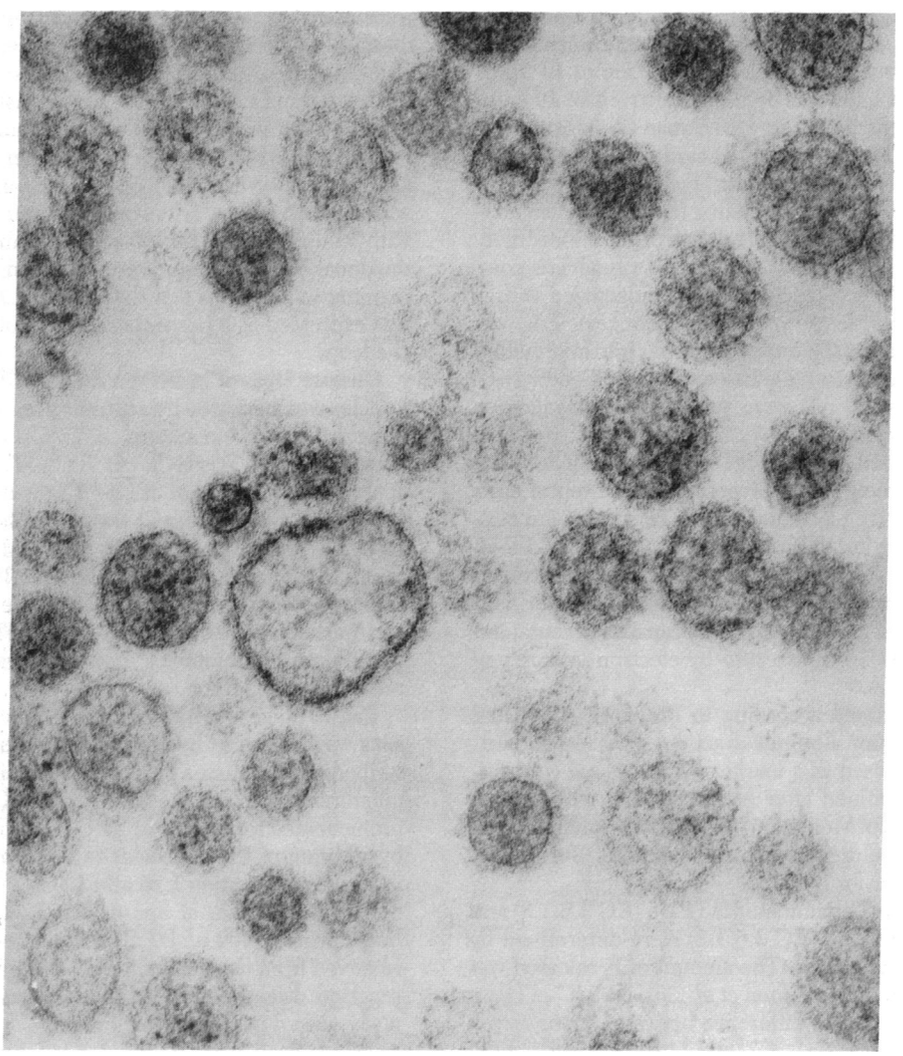

FIG. 1. Representative electron micrograph of purified brush border membranes. Homogeneity and vesiculation of the membranes are apparent. (×225,000.)

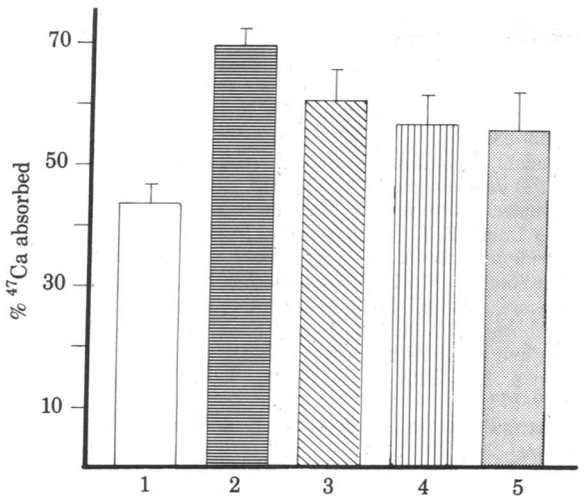

FIG. 2. Effects of 1,25-$(OH)_2D_3$ and cortisol treatment on the duodenal calcium absorption of vitamin D-deficient chicks. Experimental groups: 1, vitamin D-deficient control; 2, 1,25-$(OH)_2D_3$-treated; 3, 1,25-$(OH)_2D_3$ plus 1 mg of cortisol; 4, 1,25-$(OH)_2D_3$ plus 3 mg of cortisol; and 5, 1,25-$(OH)_2D_3$ plus 5 mg of cortisol. Number of chicks per group: 1, 14; 2, 10; 3 and 4, 11; 5, 8. Values given are the mean ± SEM. 1,25-$(OH)_2D_3$ (1 μg) was given 15 hr before sacrifice. Cortisol was given intraperitoneally 24 and 48 hr before sacrifice. Significant differences: 1 vs. 2, $P \leq 0.001$; 1 vs. 3, $P \leq 0.005$; 1 vs. 4, $P \leq 0.02$; 2 vs. 4 and 5, both $P \leq 0.05$.

within the first minute, followed by a slower uptake occurring over the next 90 min (Fig. 3). Comparison of BBM vesicle uptake between the 1,25-$(OH)_2D_3$-dosed birds and vitamin D-deficient controls revealed a 30% increase in the calcium transport in dosed birds. No significant vesicle uptake differences were evident between the 1,25-$(OH)_2$-treated and cortisol plus 1,25-$(OH)_2D_3$-treated birds at any of the time points studied (Fig. 3). Similarly, when calcium uptakes were assessed by areas under the curve, it was apparent that birds treated with 1,25-$(OH)_2D_3$ had significant increases in uptake by BBM vesicles compared to vitamin D-deficient controls ($P \leq 0.02$); also, birds

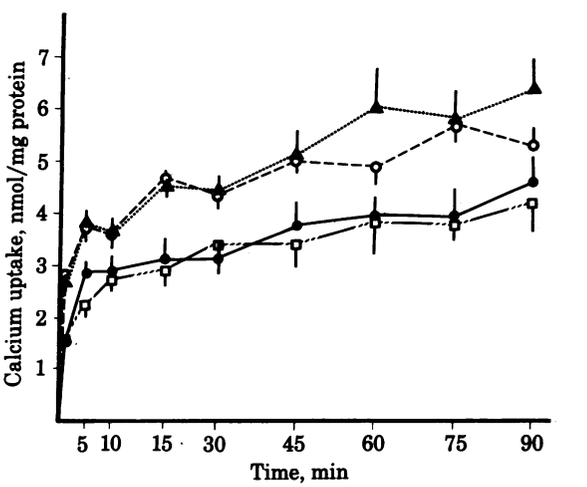

FIG. 3. Effects of 1,25-$(OH)_2D_3$ and cortisol treatment on calcium uptake by brush border membrane vesicles of vitamin D-deficient control (●; $n = 7$), 1,25-$(OH)_2D_3$-treated (▲; $n = 9$), 1,25-$(OH)_2D_3$ plus 5 mg of cortisol-treated (○; $n = 8$), and 5 mg of cortisol-treated (□; $n = 3$) chicks. Incubations were carried out in KMT buffer containing 1.25 mM $CaCl_2$. Values given are the mean ± SEM. The data represent pooled tissue from 8–10 chicks per experiment. 1,25-$(OH)_2D_3$ (1 μg) was given 15 hr before sacrifice. Cortisol was given intraperitoneally 24 and 48 hr before sacrifice.

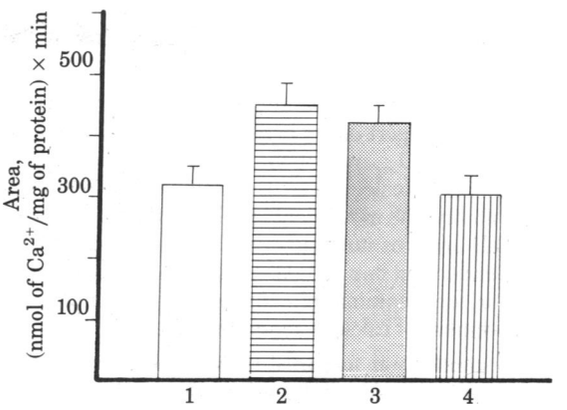

FIG. 4. Effects of 1,25-$(OH)_2D_3$ and cortisol treatment on calcium uptake by brush border membrane vesicles as assessed by areas under the curve for the different experimental groups: 1, vitamin D-deficient control ($n = 7$); 2, 1,25-$(OH)_2D_3$ ($n = 9$); 3, 1,25-$(OH)_2D_3$ plus 5 mg of cortisol ($n = 8$); and 4, 5 mg of cortisol ($n = 3$). Values given are the mean ± SEM. The data represent pooled tissue from 8-10 chicks per experiment. Significant differences: 1 vs. 2, $P \leq 0.02$; 1 vs. 3, $P \leq 0.025$; 2 vs. 4, $P \leq 0.05$; 3 vs. 4, $P \leq 0.02$.

treated with combinations of 1,25-$(OH)_2D_3$ and cortisol showed uptakes similar to the uptake of the 1,25-$(OH)_2D_3$-treated birds (Fig. 4). Cortisol alone did not diminish BBM vesicle calcium uptake in vitamin D-deficient chicks (Fig. 3 and 4).

DISCUSSION

Intestinal transport of calcium across the enterocyte first requires passage through the microvillus membrane into the cells, movement through the cell, and extrusion from the basolateral membrane. Our data show that vitamin D deficiency and cortisol treatment in the presence of 1,25-$(OH)_2D_3$ reduced *in vivo* the ability of the intestinal epithelial cell to transport calcium. Additionally, these results show that BBM vesicles prepared from 1,25-$(OH)_2D_3$-treated chicks accumulated calcium more rapidly into an intravesicular space than did the vitamin D-deficient controls. Our findings are in agreement with the observations of Rasmussen *et al.* (17), Miller and Bonner (40), and Wilson and Lawson (41), who found that 1,25-$(OH)_2D_3$ caused an increase of ^{45}Ca uptake into BBM vesicles of vitamin D-deficient rats and chicks.

In an attempt to further define the scheme of events involved in intestinal calcium transport, we studied the influence of cortisol treatment on vitamin D-mediated intestinal calcium transport, using *in situ* ligated duodenal loop calcium uptake as well as BBM vesicle uptake techniques. In agreement with a previous report (42), we found that cortisol treatment suppressed the ability of 1,25-$(OH)_2D_3$ to stimulate intestinal calcium transport *in vivo*. However, this treatment did not inhibit BBM vesicle ^{45}Ca uptake. We were thus able to dissociate BBM events from the overall transport of calcium across the intestinal cell, demonstrating that BBM vesicle calcium transport can be normal when *in vivo* calcium transport in similarly treated birds is profoundly depressed. The present results imply that intestinal mechanisms other than BBM-mediated events play an important role, at least in this model, in the movement of calcium across the intestinal epithelial cell. These mechanisms cannot be defined by the present study, however; the possibilities include new protein synthesis (15, 43, 44), mitochondrial or Golgi body transcellular calcium transport (45, 46), the basolateral cell membrane Ca^{2+}-stimulated ATPase and Na^+/Ca^{2+}-dependent ATPase extrusion pumps (47–49). One possibility, supported by the experiments of Feher and Wasserman (42) is that the

administration of cortisol in a schedule similar to the one used by us results in a significant depression in the synthesis of calcium-binding protein.

In conclusion, our observations strongly suggest that BBM events, although a part of the overall response of the intestinal cell to 1,25-$(OH)_2D_3$, are not the only event(s) of importance in the movement of calcium across the enterocyte in this model. In all probability, the movement of calcium across the duodenal mucosal cell involves multifactorial processes and may not be solely dependent on one cellular event. It is likely that protein synthesis or contraluminal phenomena play an important role in the movement of calcium across the cell.

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