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Calcium Transport by Plasma Membranes of Enterocytes during Development: Role of 1,25-$(OH)_2$ Vitamin D_3

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ABSTRACT. Calcium transport across the intestinal enterocytes represents an entry process at the brush border membranes and an ATP-dependent exit process located at the basolateral membranes. Both processes exhibit developmental changes. The present studies were designed to define the role of vitamin D in calcium transport during maturation. Brush border and basolateral membranes from vitamin D-deficient suckling and adolescent rats were used to study calcium entry and exit. 1,25-$(OH)_2$ vitamin D_3 administration enhanced calcium entry at the brush border membranes of suckling and adolescent rats. The increase in calcium uptake in both age groups was secondary to an increase in maximal transport capacity (V_{max}) rather than in K_m. In suckling rat brush border membranes, 1,25-$(OH)_2$ vitamin D_3 treatment increased the V_{max} from 1.0 ± 0.1 to 1.8 ± 0.2 nmol/mg protein/7 s ($p < 0.01$), whereas in adolescent rats, V_{max} increased from 1.5 ± 0.1 to 2.5 ± 0.3 nmol/mg protein/7 s ($p < 0.01$). K_m values were not altered. Similarly, 1,25-$(OH)_2$ vitamin D_3 administration enhanced ATP-dependent calcium exit at the basolateral membranes of both suckling and adolescent rats. V_{max} of ATP-dependent calcium uptake by basolateral membranes of suckling rats increased from 0.5 ± 0.05 to 0.81 ± 0.06 nmol/mg protein/20 s ($p < 0.01$) whereas in adolescent rats, V_{max} increased from 0.3 ± 0.03 to 0.6 ± 0.04 nmol/mg protein/20 s ($p < 0.001$). K_m values were not altered. The current studies indicate that 1,25-$(OH)_2$ vitamin D_3 stimulates calcium entry and exit across the enterocytes during maturation. (*Pediatr Res* 24:338–341, 1988)

Abbreviations

EGTA, ethylene glycol bis (B aminoethylether)-N,N,N′N,N′-tetracetic acid
HEPES, 4-(2-hydroethyl)-1-piperazinepropane-sulfonic acid
1,25-$(OH)_2$ D_3, 1,25-$(OH)_2$ vitamin D_3
BBMV, brush border membrane vesicles
V_{max}, maximal transport capacity

Calcium is an essential cation required to maintain important cellular functions and bone formation especially during periods of active growth. Our laboratory has defined the overall picture of calcium transport during maturation using *in vivo* perfusion techniques and *in vitro* everted gut sacs (1, 2). These studies have shown that during the suckling period, a high affinity low capacity system for calcium transport exists, which evolves to low affinity, high capacity system during the adolescent period (2). Moreover, a large passive component of transport exists in early life which decreases with increasing age (1, 2). These studies provided an overall view of the total transport process. Vitamin D administration to normal suckling and adolescent rats had no effect on *in vivo* calcium transport (3). However, in vitamin D-deficient adults rats, vitamin D administration enhanced the transport of calcium when studied by *in vivo* and *in vitro* everted gut sacs (4, 5). Recently, the effect of vitamin D on calcium transport was shown to be related to three major events, an increase in the entry process of calcium across brush border membranes (6), regulation of the transcellular movement (7), and increase in ATP-dependent calcium translocation across basolateral membranes (8).

The role of vitamin D in calcium transport during development in the rat has received little attention. The available data suggest no role for vitamin D in suckling rats rendered vitamin D deficient by raising pups from vitamin D-deficient dams during pregnancy and lactation (9, 10). However, either *in vivo* ligated loop technique or *in vitro* whole tissue uptake were used to determine calcium transport (9, 10). In both of these techniques, the passive as well as the active component of calcium uptake are studied together as well as the process of entry, transcellular movement, and exit processes of calcium across the epithelial cells were studied combined.

To define the role of vitamin D in calcium transport during development, we designed studies to investigate calcium entry at the brush border level and exit at the basolateral membrane in suckling and adolescent vitamin D-deficient rats and in rats treated with 1,25-$(OH)_2$ D_3.

MATERIALS AND METHODS

Vitamin D deficiency in suckling rats (14–15 days old) was induced by feeding pregnant dams at 48 h after insemination; a vitamin D-deficient diet (Ca 0.4%, P 0.3%, no vitamin D, Teklad Diets, Madison, WI) and kept in the dark. Upon delivery, pups were suckled from mothers on the vitamin D-deficient diet. Both mothers and pups were kept in the dark. Vitamin D deficiency in adolescent rats (48 ± 4 days old) was induced by feeding the vitamin D-deficient diet to weanling rats for 4 wk while being kept in the dark.

One litter of suckling rats or two adolescent rats were injected intraperitoneally with Calcijex [1,25-$(OH)_2$ D_3] 100 pg/100 body weight, 8 h before death. Control vitamin D-deficient rats were injected with similar volume of the vehicle (buffered normal saline). At the time of study, rats were killed by cervical dislocation and brush border or basolateral membranes were prepared as previously described in our laboratory (11–13). Briefly, brush border membranes from mucosal homogenate were prepared by

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$MgCl_2$ precipitation technique whereas basolateral membranes were prepared from freshly harvested enterocytes with percoll density gradient. Both procedures yielded a highly enriched preparation and has been used in our laboratory. Blood was drawn from the aorta after ether anesthesia for measurement of 1,25-$(OH)_2$ D_3.

Uptake was measured by a rapid filtration technique (11–13). All experiments were performed at room temperature. Transport was initiated by adding 20 μl of the final vesicle suspension to the desired incubation media containing labeled substrate. The composition of the incubation media for each individual experiment is described in "Results." At the desired time intervals, the reaction was stopped by the addition of ice cold stop solution consisting of 100 mM mannitol, 100 mM KCl, 20 mM HEPES/Tris (pH 7.4), 5 mM $MgCl_2$, and 1 mM EGTA. ATP was added as the Tris/salt (5 mM). Free calcium concentrations less than 10 μM were maintained with EGTA buffering system as detailed by Pershadsing and McDonald (14). Typically, total calcium was held at 1 mM and EGTA was increased to yield the desired free calcium concentration.

The vesicles were immediately collected on a cellulose nitrate filter (0.45 μm pore size, Sartorius Filters, Inc., Hayward, CA) and kept under suction while being washed with ice-cold stop solution. The amount of radioactive substrate remaining on the filter was determined in a liquid scintillation counter (Beckman Instrument, Palo Alto, CA) using Scinti Verse II solution (Fisher Scientific, Norcross, GA) as a liquid scintillant. Radioactivity remaining in the filters after pipetting incubation medium into the radioactive substrate in the absence of vesicles was considered as background and used in the calculation. Results are expressed as mean ± SEM. Each experiment was run in triplicate and repeated on three separate occasions.

^{45}Ca (10–40 mCi/mg) was obtained from New England Nuclear, Boston, MA. Enzymes and substrates for leucine aminopeptidase were obtained from Sigma Chemical Corp., St. Louis, MO. Cellulose nitrate filters, 0.45 mM pore size, were obtained from Sartorius Filter, Inc., Hayward, CA. All other chemicals were of the highest purity available. Calcijex [1,25-$(OH)_2$ D_3, 1 μg/ml] was kindly provided by Abbott Laboratories (Abbott Park, IL). 1,25-$(OH)_2$ D_3 was measured using radioreceptor assay (Immuno Nuclear Corp., Stillwater, MN).

Purity of the brush border and basolateral membrane preparation was determined by marker enzyme activity. Leucine aminopeptidase activity, a marker for brush border membranes, was measured using a kit from Sigma. Na^+-K^+-ATPase, a marker for basolateral membrane was measured by the method of Scharschmidt *et al.* (15). Cytochrome oxidase and NADPH-cytochrome-C reductase markers for mitochondria and endoplasmic reticulum respectively were measured as described by Beaufy *et al.* (16).

RESULTS

Brush border membranes were enriched 12- to 14-fold with leucine aminopeptidase whereas marker enzymes of Na^+-K^+-ATPase, cytochrome C-oxidase and NADPH-cytochrome-C reductase were all impoverished. Basolateral membrane vesicles were enriched 10- to 12-fold with Na^+-K^+-ATPase whereas marker enzymes of leucine aminopeptidase cytochrome-C-oxidase and NADPH-cytochrome-C reductase were all impoverished. Enrichment and impoverishment of marker enzymes were similar in both suckling and adolescent rats.

Table 1 depicts serum concentration of 1,25-$(OH)_2$ D_3 in control rats, vitamin D-deficient rats, and in vitamin D-deficient rats injected with 1,25-$(OH)_2$ D_3. As seen, 1,25-$(OH)_2$ D_3 was significantly lower compared to controls. Moreover, 1,25-$(OH)_2$ D_3 administration resorted serum 1,25-$(OH)_2$ D_3 to normal levels.

Calcium uptake by BBMV. BBMV were preloaded with 280 mM mannitol and 20 mM HEPES/Tris pH 7.4. Incubations were conducted at 25° C in a media containing 100 mM NaCl, 100 mM mannitol, 20 mM Hepes/Tris pH 7.4, 0.1 mM $CaCl_2$, and tracer ^{45} Ca. Calcium uptake was determined with time. As seen in Figure 1, calcium uptake in both suckling and adolescent rats treated with 1,25-$(OH)_2$ D_3 was significantly greater compared to vitamin D-deficient rats at all time points studied. The magnitude of stimulation was more marked with increasing time in both suckling and adolescent rats.

To determine the mechanism for the increased uptake with 1,25-$(OH)_2$ D_3 treatment, kinetic studies were conducted at 7 s. In previous studies, we have shown that calcium uptake was linear up to 15 s (11). As seen in Figure 2, V_{max} of calcium uptake was significantly greater in animals treated with 1,25-$(OH)_2$ D_3 suggesting that the capacity to transport calcium is increased without a change in the affinity.

Calcium transport by basolateral membrane. Basolateral membrane vesicles were brought up in 280 mM mannitol and 20 mM HEPES/Tris, pH 7.2 buffer. Incubations were initiated in a media containing 100 mM mannitol, 100 mM KCl, 5 mM Mg ATP, 20 mM HEPES/Tris pH 7.2, 0.1 mM $CaCl_2$, and tracer ^{45}Ca. As seen in Figure 3, ATP-driven calcium uptake in both suckling and adolescent rats treated with 1,25-$(OH)_2$ D_3 was significantly greater compared with corresponding values in vitamin D-deficient rats.

For kinetic studies, basolateral membrane vesicles were brought up in 280 mM mannitol, 20 mM HEPES/Tris pH 7.2 buffer. Incubations were initiated in a media containing 100 mM mannitol, 100 mM KCl, 5 mM Mg ATP, 20 mM HEPES/Tris buffer, pH 7.2, and different calcium concentrations to range between 0.01–2.5 μM (μM calcium concentration was maintained by Ca-EGTA buffering system). Calcium uptake was

Table 1. *Serum concentration of 1,25-$(OH)_2$ D_3 in control rats, vitamin D-deficient rats, and in vitamin D-deficient rats injected with 1,25-$(OH)_2$ D_3**

	Control	Vitamin D deficient	Vitamin D deficient injected with 1,25-$(OH)_2$ D_3
Suckling rats	80 ± 6	12 ± 5	93 ± 10
Adolescent rats	157 ± 2	41 ± 10	192 ± 12

* Values are pg/ml and represent mean ± SEM (n = 6); samples from suckling rats were pooled (each pooled samples represents one litter).

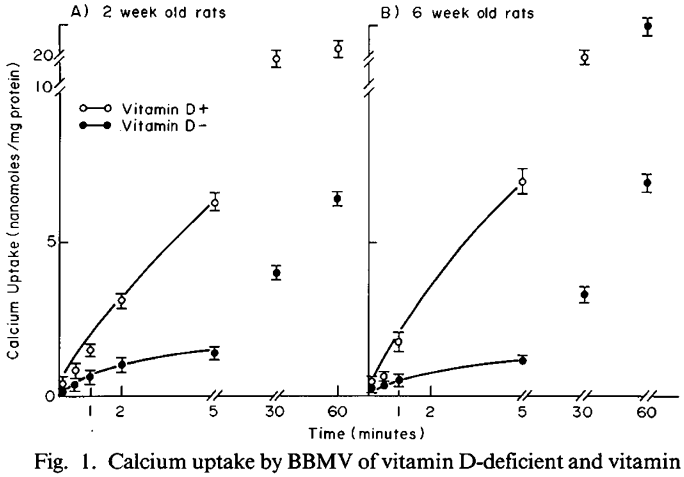

Fig. 1. Calcium uptake by BBMV of vitamin D-deficient and vitamin D-treated suckling and adolescent rats. Intestinal BBMV from adolescent and suckling rats were preloaded with 280 mM mannitol and 20 mM HEPES/Tris, pH 7.4. Incubations were conducted at 25° C in a medium containing either 100 mM NaCl, 100 mM mannitol, 20 mM HEPES/Tris, pH 7.4, and 0.1 mM $^{45}CaCl_2$. Values are mean ± SEM of (n = 6) determinations. Uptake values in vitamin D-treated rats were significantly more than in vitamin D-deficient rats ($p < 0.05$–0.001).

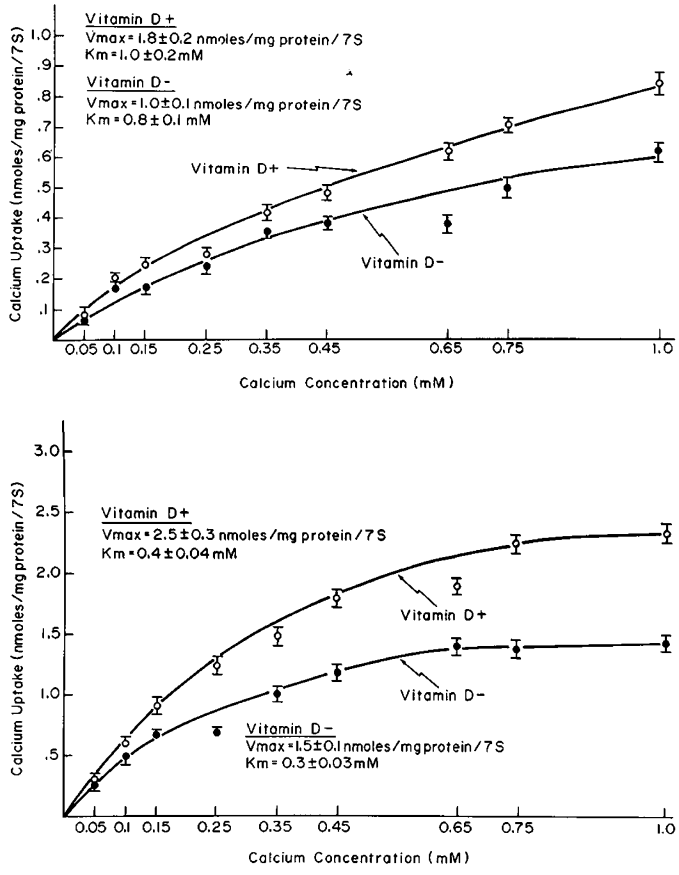

Fig. 2. Kinetics of calcium uptake by BBMV of vitamin D-deficient and vitamin D-treated suckling (*A*) and adolescent rats (*B*). Intestinal BBMV were brought up in mannitol buffer, pH 7.4. Calcium uptake was determined at 7 s. Calcium concentration in the media ranged from 0.05–1 mM. Kinetic parameters were obtained using a computerized model of the Michaelis-Menten. V_{max} values for vitamin D-deficient and vitamin D-treated suckling and adolescent rats were significantly different ($p < 0.02$).

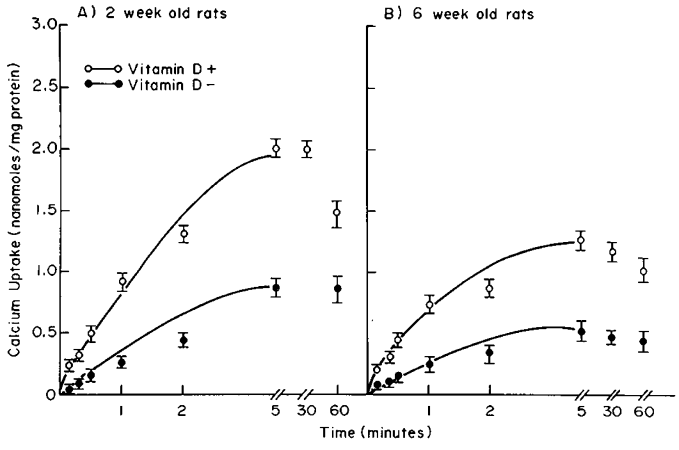

Fig. 3. Calcium uptake by basolateral membrane vesicles of vitamin D-deficient and vitamin D-treated suckling and adolescent rats. Intestinal basolateral membrane were incubated in a media containing 100 mM mannitol, 100 mM KCl, 5 mM $MgCl_2$, 120 mM HEPES/Tris, pH 7.4, 5 mM ATP, and 0.1 mM $^{45}CaCl_2$. Reaction was stopped at desired time points. Values are mean ± SEM of two experiments. Each experiment was run in triplicate. Uptake values in vitamin D-treated animals were significantly more ($p < 0.05$–0.001) compared to corresponding values in vitamin D-deficient animals.

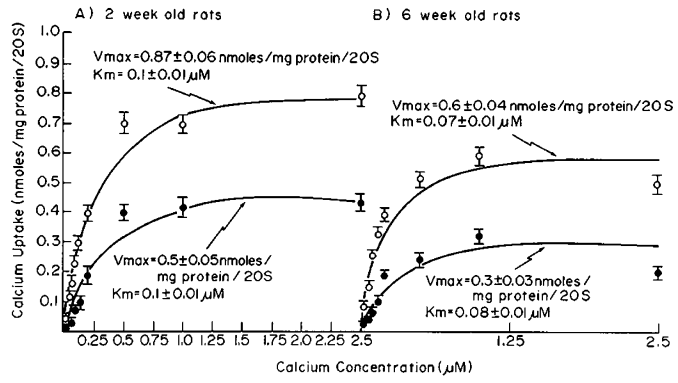

Fig. 4. Kinetics of calcium uptake by basolateral membrane vesicles of vitamin D-deficient and vitamin D-treated suckling and adolescent rats. Intestinal basolateral membranes were incubated in a media containing 100 mM mannitol, 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES/Tris buffer, pH 7.4, in the absence or presence of 5 mM ATP-Tris salt and different calcium concentration 0.01–2.5 μM with tracer ^{45}Ca. Micromolar concentrations of calcium were achieved by EGTA buffering system as described by Pershadsing and McDonald (14). Kinetic parameters (K_m and V_{max}) were calculated from uptake values with ATP minus no ATP using a computerized model of Michaelis-Menten kinetics. V_{max} values for vitamin D-deficient and vitamin D-treated suckling and adolescent rats were significantly different ($p < 0.01$).

determined at 20 s within the linear line of uptake. As seen in Figure 4, V_{max} of ATP driven calcium uptake increased by 1,25-$(OH)_2$ D_3 treatment in both suckling and adolescent rats while K_m remained similar.

DISCUSSION

The data presented describe the effect of 1,25-$(OH)_2$ D_3 treatment on calcium transport by BBMV and basolateral membranes of intestinal enterocytes from vitamin D-deficient suckling and adolescent rats. 1,25-$(OH)_2$ D_3 enhanced calcium uptake at the BBMV and basolateral membranes of both age groups, by increasing the capacity of the uptake process. Previous studies in adult animals with vitamin D deficiency has clearly shown that vitamin D administration stimulated calcium transport *in vivo* and *in vitro* (4, 5). The actions of vitamin D on calcium transport has been shown to be related to *1*) increase in the entry process at the BBMV level (6); *2*) induction of calcium binding protein that may facilitate calcium entry and movement across the cytoplasm (17); and *3*) increase in Ca^{++}-Mg^{++} ATPase pump activity with stimulation of calcium extrusion at the basolateral membrane (8).

During the suckling period in the rat (14–15 days), the majority of calcium is transported by a nonsaturable component (1, 2). The active transport process constitutes approximately 20% of total uptake (2). This active component is not expressed until the rat is about 10 days old (18). Previous studies on the role of vitamin D in calcium transport in the suckling rat showed no stimulation of calcium uptake using *in situ* loop technique (10) or *in vitro* whole tissue uptake (9). However, these two techniques measure a composite of calcium transport processes that includes a major passive component as well as entry across the brush borders, transcellular movement, and exit at the basolateral membrane. Therefore, it is possible that a specific effect on a single site of calcium movement could be masked. Moreover, it is known that the passive calcium movement that constitutes a large proportion of calcium movement in the suckling rats, is not under the influence of vitamin D in adult animals.

Our studies are unique because they address using isolated membrane vesicles and the role of vitamin D at the entry and exit steps. Autoradiographic studies have shown the presence of 1,25-$(OH)_2$ D_3 receptors in the crypt cells as early as 2–3 days after birth and by 10 days of age, nearly all crypt cells appear to have receptors. Receptor concentration is detectable biochemically during the suckling period and appears to reach a maximum at 18 days of age (19). Moreover, Ueng *et al.* (20) showed that 1,25-$(OH)_2$ D_3 treatment in 12-day-old pups increased the level of Ca^{++} binding protein by 2-fold. Furthermore, as seen in Table 1, 1,25-$(OH)_2$ D_3 is present in suckling rats. These observations suggest that suckling rats at 14–15 days of age may respond to 1,25-$(OH)_2$ D_3 treatment under appropriate experimental conditions, *i.e.* with plasma membrane fractions.

As has been shown in adult animals, vitamin D enhanced calcium transport across the BBMV of the suckling and adolescent rats. This finding is consistent with the observation of several investigators (6, 20) and suggest that vitamin D increases the permeability of the brush border membrane to calcium. In this regard, it is interesting to note that Putkey and Norman (22) have presented evidence to suggest that vitamin D status affects the composition and topology of the BBMV protein as well as its associated cytoskeleton core proteins that are important in calcium transport.

The active step in calcium transport is believed to be located at the basolateral membrane (8). An ATP-dependent calmodulin-regulated mechanism has been described in the basolateral membranes of the enterocyte (23). Recently, we have described a similar mechanism in suckling rat basolateral membranes (12). Our current data suggest that vitamin D administration enhanced the V_{max} of the ATP-dependent calcium transport system in the basolateral membranes of the suckling and adolescent rats. This observation is consistent with the observation of Ghisjen and Van Os (8). More recently, Van Corven *et al.* (24) have shown that the method of cell isolation used to prepare basolateral membrane may influence the response to vitamin D. In basolateral membranes isolated by the citrate buffer, as in our studies, vitamin D deficiency decreased duodenal calcium uptake by 5-fold. However, when basolateral membranes were prepared from cells isolated by vibration method, no effect of vitamin D deficiency was noted. The authors concluded that ATP driven calcium pump in duodenal plasma membranes from vitamin D-deficient rats, is more prone to inactivation during enterocyte isolation procedures (24). These observations imply that vitamin D deficiency alters membrane composition. However, studies that address membrane composition are lacking. In summary, our results indicate an effect of 1,25-$(OH)_2$ vitamin D_3 on calcium transport across BBMV and basolateral membrane vesicles during development in the rat. These observations are consistent with the multiple actions of vitamin D on calcium transport processes across the intestinal enterocyte.

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