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Intestinal Maturation: Calcium Transport by Basolateral Membranes

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ABSTRACT. The overall characteristics of calcium transport across the intestine have been defined using *in vivo* perfusion techniques and *in vitro* everted gut sacs. However, calcium transport represents three separate processes: entry at the brush border membranes, movement across the cytoplasm, and exit at the basolateral membranes (BLM). Studies in adult animals indicated that the active step in calcium transport is located at the BLM. The present studies describe for the first time the maturational aspects of calcium transport across the BLM of the enterocyte. We utilized a percoll density gradient to prepare enriched BLMs from suckling and adolescent rats. Calcium uptake into BLMs represented mainly transport into the intravesicular space. Calcium transport in both age groups was driven by ATP; the calcium transport in the presence and absence of ATP was significantly greater in suckling rats compared to adolescent rats. Kinetics of calcium uptake calculated from uptake values in the presence of ATP minus no ATP conditions showed a K_m of 0.06 ± 0.01 and 0.03 ± 0.01 μM for adolescent and suckling rats respectively ($p < 0.05$). V_{max} was 1.5 ± 0.1 and 0.8 ± 0.08 nmol/mg protein/min for adolescent and suckling rats respectively ($p < 0.01$). Calcium/sodium exchange mechanisms was also present in both age groups. However, the magnitude of sodium-dependent calcium exchange was smaller in suckling rats compared to adolescent rats. These data suggest that calcium exit at the BLMs of enterocytes of suckling and adolescent rats occurs by an ATP-dependent and a calcium/sodium exchange mechanism. Both mechanisms exhibit maturational changes. (*Pediatr Res* 21: 257–260, 1987)

Abbreviation

BLMV, basolateral membrane vesicles

Calcium is an essential element 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 technique 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 evolve 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 provide an overall view of the total transport process. However, calcium transport represents three separate processes: entry at the brush border membrane, movement across the cytoplasm, and exit at the basolateral membranes. Because the cell interior is negative to both mucosa and serosal compartments and since calcium concentrations in the intracellular and extracellular fluid are <1 μM and 1 mM, respectively, active steps of calcium transport have to be expected in the basolateral plasma membrane of the enterocyte (3). A calcium/sodium exchange mechanism was suggested, based on the Na-dependence of serosal Ca-efflux from the epithelium (4). An ATP-dependent calcium process has also been found in basolateral membranes of enterocyte and kidney tubules (3, 5, 6). These concepts are in agreement with calcium transport systems in a variety of other plasma membranes (7). Although these studies have characterized these processes in adult animals, virtually no information is available on how Ca^{++} exit from the enterocyte during maturation. Because the overall picture of calcium transport evolves with age, we hypothesized that developmental changes in calcium transport by basolateral membranes may also occur.

The current study is designed to characterize calcium transport by basolateral membranes of suckling and adolescent rats. We have utilized basolateral membrane vesicles prepared by a Percoll density gradient in which Na^+-dependent D-glucose transport characteristic of brush border membrane vesicles could not be measured.

MATERIALS AND METHODS

Materials. D-[^{3}H] glucose (18.1 Ci/mmol) and ^{45}Ca (10–40 mCi/mg) were obtained from New England Nuclear Corp., Boston, MA. Valinomycin, enzymes, and substrates for leucine amino peptidase were obtained from Sigma Chemical Corp., St. Louis, MO. Cellulose nitrate filters, 0.45 μm pore size, were obtained from Sartorius Filter, Inc., Haywood, CA. All other chemicals were of the highest purity available.

Preparation of BLMV. Jejunal BLMV were prepared using a modified centrifugation technique followed by separation on a Percoll gradient (8). Two jejunums of adolescent rats or 10 jejunums of suckling rats were used for each preparation. The intestinal segments were removed, flushed with ice cold lactated Ringer's solution, and filled with warmed buffer solution (37° C) containing 1.5 mM KCl, 96 mM NaCl, 8 mM KH_2PO_4, 27 mM Na_3 citrate, and 2 mM dithiothreitol (pH 7.4) (buffer I). The segments were then clamped and incubated for 15 min in a shaking water bath at 37° C. The clamps were removed and the contents emptied. The segments were then filled with ice cold buffer containing 100 mM mannitol, 100 mM KCl, 24 mM Hepes/Tris buffer pH 7.4 (buffer II) and gently palpated by fingers for 5 min to release epithelial cells. The contents were drained into a beaker on ice and the volume was brought up to 250 ml in buffer II. The cells were then centrifuged at 200 × *g* for 5 min and the cell pellet was homogenized in 125 ml buffer II in an Omni mixer for 3 min. The homogenate was then

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brought up to 150 ml with buffer II and centrifuged at 22,000 × *g* for 25 min. The supernatant was discarded and the resulting fluffy layer of the pellet was resuspended in 95.5 ml of buffer II and homogenized in a glass-Teflon homogenizer (20 strokes). The resultant homogenate was mixed with Percoll (Pharmacia, Piscataway, NJ) at a concentration of 12.5% and centrifuged at 48,000 × *g* for 65 min. A distinct band of basolateral membranes was seen at the upper one-third of the Percoll gradient. The band was aspirated by a needle and the pellet resuspended in the appropriate transport buffer. The resuspended pellet was then centrifuged at 48,000 × *g* for 20 min and finally suspended in 100 mM KCl, 100 mM mannitol, 5 mM $MgCl_2$, and 20 mM Hepes/Tris buffer (pH 7.4).

Transport measurements. Uptake of substrates (D-glucose, calcium) was measured by a rapid filtration technique (9). All experiments were preformed 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. For glucose studies 0.2 mM phloretin was also added. ATP was added as the tris/salt (5 mM) with ^{45}Ca. Free calcium concentrations less than 10 μM were maintained with EGTA buffering system as detailed by Pershadsingh and McDonald (10). 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 micrometer 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 ± SE. Each experiment was run in triplicate and repeated on three separate occasions.

Purity of vesicle preparation. Purity of the basolateral membrane preparation was determined by marker enzyme activity. Leucine amino peptidase activity a marker for brush border membranes was measured using a kit from Sigma. Disaccharidases, another marker for brush border enzymes, were measured as described by Dahlqvist (11). Na^+-K^+-ATPase, a marker for basolateral membrane, was measured by the method of Scharschmidt *et al.* (12). Cytochrome oxidase and NADPH-cytochrome-C reductase markers for mitochondria and endoplasmic reticulum, respectively, were measured as described by Beaufy *et al.* (13). Galactosyl transferase, a marker for golgi apparatus, was measured as described by Morre *et al.* (14).

RESULTS

Purity of membrane vesicles. Marker Enzyme Studies. The specific activity of Na^+-K^+-ATPase, a marker for basolateral membranes, was enriched 9 ± 2 times as compared to mucosal homogenate. Leucine aminopeptidase and disaccharidases (brush border membrane markers) were 0.9 ± 0.2 times, cytochrome-c-oxidase (mitochondrial membrane marker) 0.6 ± 0.1 times, and NADPH-cytochrome-c-reductase (endoplasmic reticulum marker) 0.7 ± 0.1 times. Galactosyl transferase (golgi apparatus) was not detectable compared to crude homogenate activity of 20 ± 3 nmol/h/mg protein. Enrichment and impoverishment of marker enzymes were similar in both suckling and adolescent rat basolateral membranes.

Orientation of the Basolateral Membrane. The orientation of the basolateral membrane vesicles was determined by the latency of the enzyme activity in the Na^+-K^+-ATPase. Basolateral membranes were measured before and after treatment of 0.06% deoxycholate and 1.5 mM EDTA. As seen in Table 1, 50–59% of the vesicles are present as inside out vesicles in both suckling and adolescent rats, respectively.

D-Glucose uptake by BLMV. Figure 1 depicts D-glucose uptake in the presence and absence of Na^+ gradient. Transient accumulation of D-glucose above equilibrium, a characteristic of brush border membrane vesicles, was not observed. Moreover,

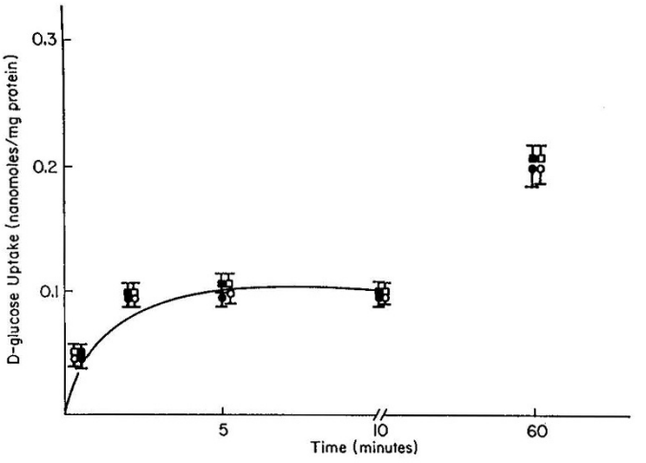

Fig. 1. D-Glucose uptake by intestinal BLMV. Intestinal BLMV of suckling (*circles*) and adolescent (*squares*) rats were incubated in a media containing either 100 mM NaCl (*closed symbols*) or 100 mM KCl (*open symbols*), 100 mM Mannitol, 20 mM Hepes-Tris buffer pH 7.4, and 0.1 mM D-glucose with ^3H-glucose as a tracer. At desired time points (30 s, 2, 5, 10, and 60 min) reaction was stopped by diluting the vesicles in ice cold stop solution. Values as mean of three separate experiments. Each experiment was run in triplicate.

Table 1. *Effect of treatment with deoxycholate/EDTA on mg ATPase and Na-K-ATPase in basolateral membranes; ATPase activity is expressed as nmol Pi/mg protein/min**

	Mg + Na + K + ouabain	Incubation medium Mg + Na + K	Na − K ATPase	%
Adolescent				
−Deoxycholate/EDTA	180 ± 30	230 ± 60	50	41 ± 3
+Deoxycholate/EDTA	210 ± 10	330 ± 60	120	100
Suckling				
−Deoxycholate/EDTA	380 ± 30	490 ± 30	90	50 ± 4
+Deoxycholate/EDTA	510 ± 60	690 ± 40	180	100

* Values are ± SE and represent three different determinations. BLMV were treated with a mixture of deoxycholate and EDTA (final concentration 0.06 and 1 mM, respectively) for 30 min at room temperature. Immediately after this treatment the samples were analyzed for enzyme activities. Mg concentration 5 mM, Na 100 mM, K 20 mM, and ouabain 1 mM.

there was no difference in uptake in the presence of Na^+ or K^+ gradients. Intravesicular volume calculated for D-glucose equilibrium values was 2.1 ± 0.2 μl/mg protein for both suckling and adolescent rats. Similar intravesicular volumes were obtained using ^{14}C mannitol uptake values at equilibrium (data not shown).

Osmolarity. To determine whether calcium uptake is transported into osmotically active spaces, or represents mere binding, basolateral membranes from suckling and adolescent rats were incubated in solutions with various osmolarities, and calcium uptake was determined at equilibrium (120 min). Figure 2 depicts calcium uptake in relationship to media osmolarity in the suckling rat. The majority of uptake represented uptake into the intravesicular space ($Y = 0.032 \times + 0.34$). Therefore at isotonicity 25% of uptake represents binding. A similar relationship exists for membrane of adolescent rats.

ATP Driven Calcium Uptake with Time. Figure 3 depicts calcium uptake by basolateral membranes in both suckling and adolescent rats. In suckling and adolescent basolateral membranes, the presence of ATP energized uphill transport of calcium compared to no ATP conditions. However, in the absence of ATP, calcium uptake was significantly greater in suckling basolateral membranes compared to corresponding values in adolescent rats. Maximal calcium uptake at 10 min was significantly greater in suckling rats compared to adolescent rats. Calcium release occurred after 10 min in both age groups.

Kinetics of calcium uptake. Kinetics of calcium uptake was calculated from the difference between uptake in the presence and absence of ATP. Figure 4 depicts the kinetic parameters for calcium uptake in suckling and adolescent basolateral membranes. Kinetics were determined at 1 min, well within the linear line of uptake. K_m and V_{max} were calculated by a computerized model of the Michaelis-Menten kinetics. V_{max} values were 1.5 ± 0.1 and 0.8 ± 0.08 nmol/mg protein/min for adolescent and suckling rats, respectively, while K_m values were 0.06 ± 0.01 and 0.03 ± 0.01 μM for adolescent and suckling rats, respectively. V_{max} and K_m values for both age groups were significantly different ($p < 0.05$–0.01).

Na^+/Ca^{++} exchange. The presence of Na^+/Ca^{++} exchange system was investigated in two ways. First, sodium was added to the incubation media at a concentration of 50 mM by replacing equimolar concentration of Na^+ with K^+. As seen in Figure 3, the presence of Na^+ inhibited ATP-dependent Ca^{++} uptake in both suckling and adolescent rats; however, the magnitude of the inhibition was higher in adolescent rats compared to suckling rats. In a second series of experiments Na^+/Ca^{++} exchange was determined by Ca^{++} efflux studies in the presence of KCl or NaCl. BLMV were incubated in calcium-containing media for 15 min. Efflux was then determined. As seen in Figure 5, Ca^{++} efflux was more rapid in suckling rats compared to adolescent rats. Ca^{++} efflux in the presence of Na^+ was significantly greater than in the presence of KCl ($p < 0.05$) in both age groups. However, the magnitude of Na^{++}-dependent Ca^{++} efflux was significantly greater in adolescent rats compared to suckling rats.

The effect of mitochondrial inhibitors on ATP driven calcium uptake. Mitochondrial ATP-dependent Ca uptake is known to

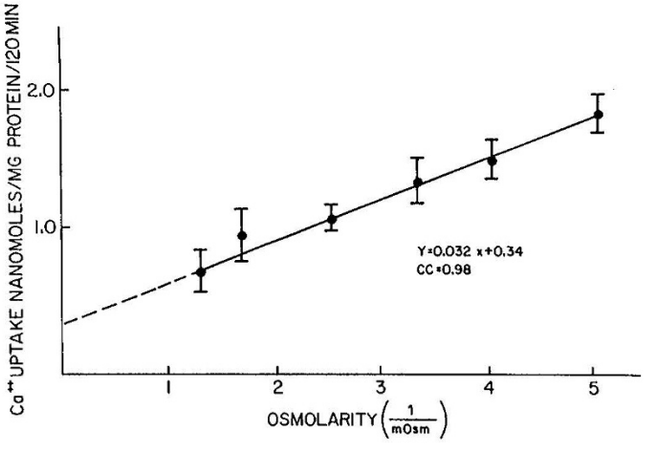

Fig. 2. Effect of media osmolarity on calcium uptake. Intestinal BLMV from suckling rats were incubated in a media containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP-tris salt, 20 mM Hepes-Tris buffer pH 7.4, 0.1 mM $CaCl_2$, and ^{45}Ca as a tracer and various concentration of mannitol to achieve osmolarities between 200–700 mOsmol. At 120 min reaction was stopped by diluting vesicles in an ice cold stop solution. Values are mean of three separate experiments. Each experiment was run in triplicate.

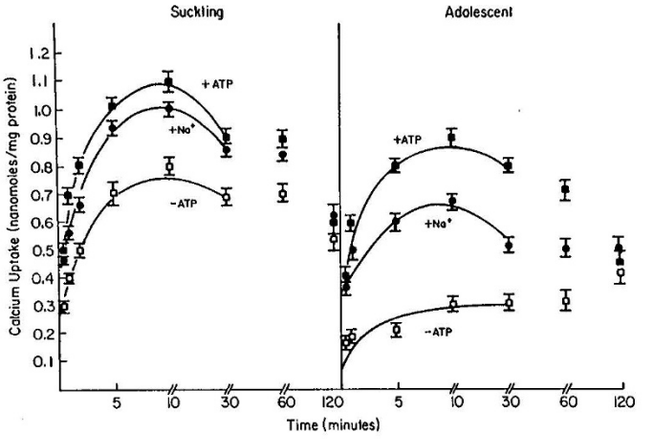

Fig. 3. ATP driven calcium uptake. Intestinal basolateral membranes of suckling and adolescent rats were incubated in a media containing 100 mM mannitol, 100 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes-Tris buffer, 0.1 mM $CaCl_2$, and tracer ^{45}Ca in the absence (*open squares*) or in the presence of 5 mM ATP Tris salt (*closed squares*). In some experiments 50 mM NaCl was substituted for 5 mM KCl (*closed circles*). The reaction was stopped at desired time intervals. Values are mean of three separate experiments. Each experiment was run in triplicate.

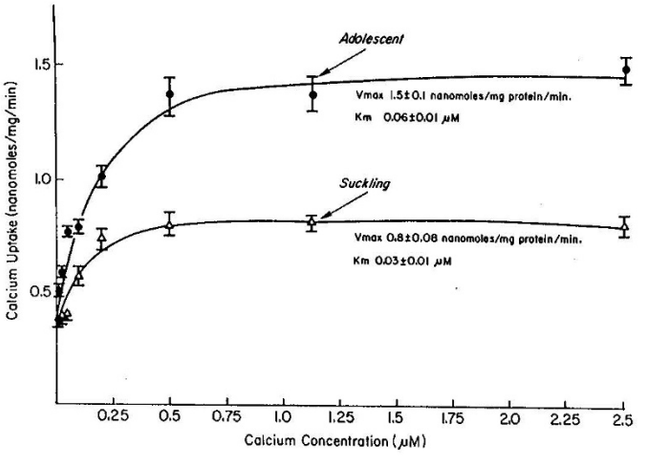

Fig. 4. Kinetics of calcium uptake. Intestinal basolateral membranes of suckling and adolescent rats were incubated in a media containing 100 mM Mannitol, 100 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes-Tris Buffer pH 7.4 in the absence or presence of 5 mM ATP-tris salt and different calcium concentration 0.01–2.5 μM with tracer ^{45}Ca. Micromolar concentrations of calcium were achieved by EGTA buffering system as described by Pershadsingh and McDonald (10). 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} and K_m values for suckling and adolescent rats were significantly different ($p < 0.01$).

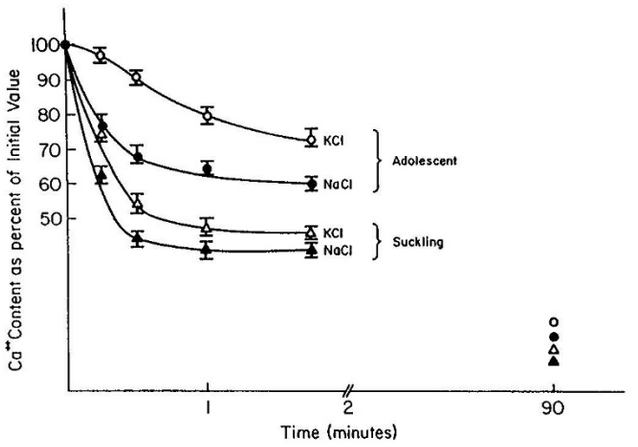

Fig. 5. Calcium/sodium exchange. Intestinal BLMV from suckling (*triangles*) and adolescent (*circles*) rats were loaded for 15 min with 0.1 mM $CaCl_2$ and tracer ^{45}Ca in the presence of 100 mM mannitol, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP-tris salt, and 20 mM Hepes-Tris buffer pH 7.4. The reaction was started by diluting loaded vesicles in a media containing either 100 mM mannitol, 20 mM Hepes-Tris buffer pH 7.4 with 100 mM KCl (*open symbols*) or 100 mM NaCl (*closed symbols*). Samples were removed at desired time intervals. Values were expressed as percent initial values at time 0. Each experiment was run three times in triplicate.

be inhibited by oligomycin (10 μg/ml). This inhibitor was tested in our system. The addition of oligomycin 10 μg/ml to the incubation media did not inhibit ATP-dependent calcium uptake in both age groups suggesting that calcium uptake represents a property of the basolateral membranes.

DISCUSSION

The present studies are the first to characterize calcium transport across basolateral membranes during maturation. Our basolateral membranes were prepared by an established technique utilizing percoll density gradient. To be able to compare rates of transport in suckling and adolescent basolateral membranes, we first characterized our preparation in both age groups. Enzyme enrichment criteria in both age groups indicated an enriched preparation with Na^+-K^+-ATPase as a marker for basolateral membranes while markers for brush border membranes, mitochondria, and endoplasmic reticulum were impoverished. The orientation of the vesicle preparation in both age groups was also similar. Moreover, vesicle size from D-glucose and mannitol data was 2.1 ± 0.02 μl/mg protein for both suckling and adolescent preparations. These studies coupled with absence of "overshoot" phenomena for D-glucose in basolateral membrane of both age groups suggest no contamination with brush border membranes. Based on these studies our basolateral membranes appeared suitable to carry out the proposed studies. Figure 2 provides evidence that an ATP-driven calcium transport system exists in both suckling and adolescent rats preparations. Because the mitochondria possesses an ATP-dependent calcium uptake system, we investigated whether contamination with mitochondria had occurred in our preparation. Oligomycin is a specific inhibitor for mitochondria. ATPase activity did not inhibit Ca^{++} uptake in our preparation (3). Therefore, ATP-dependent Ca^{++} uptake in our preparation represents a property of the basolateral membranes. The difference between ATP-dependent and ATP-independent uptake was greater in the adolescent rats compared to the suckling rats. The maximal uptake in both age groups was noted to be at 10 min. The kinetic parameters showed a V_{max} of 1.5 ± 0.1 nmol/mg protein/min and a K_m of 0.06 ± 0.01 μM in the adolescent rats. These values were two times greater than corresponding values obtained for suckling rats ($p < 0.01$). Thus it appears that suckling basolateral membranes possess a high affinity low capacity system compared to high capacity low affinity system in the adolescent rats. It is important to note that the experimental procedure was designed to investigate transport at a concentration (μM) of calcium to simulate physiological conditions. Moreover the kinetic data represent insideout vesicle since only these vesicles then have a ATP site accessible. Therefore, the true V_{max} may be higher. The transport affinity of the intestinal system is less than values reported for free cytosolic calcium concentrations, thus providing the pump with the required affinity at such physiological calcium activities. The energy required for uphill transport of calcium is provided by the hydrolysis of ATP localized at the basolateral membrane. Work by Ghijsen and Os (15) provided evidence for the presence of Mg^{++}-ATPase which is the enzymatic expression of the transport system. In the next series of experiments we characterized the Na^+/Ca^{++} exchange system in both age groups. Influx studies in the presence of Na^+ as well as efflux studies in the presence of Na^+ or K^+ clearly indicate that a Na^+/Ca^{++} exchange is present. In the influx studies the presence of Na^+ inhibited Ca^{++} uptake while in the efflux studies the presence of Na^+ in the incubation media resulted in enhanced release of Ca^{++} compared to situation where K^+ is present. These studies indicated the presence of a Na^+/Ca^{++} exchange system in our preparation. However, in a quantitative manner the effect of Na^+ was less pronounced in the suckling rat compared to the adolescent rat. These findings indicate that Na^+/Ca^{++} exchange plays a minor role in translocation of Ca^{++} across the basolateral membrane of the suckling rat. This process shows maturational changes as evidenced by the greater effect of Na^+ on the Na^+/Ca^{++} exchange system. The findings of two transport systems for calcium across the basolateral membrane are similar to those described for rat renal proximal tubular basolateral membrane (5, 6).

In summary, the present studies suggest an ATP driven and a Na^+/Ca^{++} exchange system in both suckling and adolescent rats; however, these two mechanisms show maturational changes that evolve with age.

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