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Developmental Maturation of Calcium Transport by Rat Brush Border Membrane Vesicles

FAYEZ K. GHISHAN AND NOUSHIN ARAB

Vanderbilt University, Department of Pediatrics, Division of Gastroenterology, Medical Center North D-4130, Nashville, Tennessee 37232

ABSTRACT. The developmental aspects of calcium transport across the intestinal brush membrane vesicles was studied utilizing a millipore filtration technique. Calcium transport represented uptake into the intravesicular space as evidenced by osmolality studies, calcium release by the calcium ionophore A23187, and temperature dependency. Calcium transport in both suckling and adolescent rats appears to occur by a saturable mechanism. Calcium uptake was similar in the presence of sodium and potassium gradients, but decreased in the presence of choline gradient. The imposition of negative membrane potential did not enhance calcium uptake compared to voltage clamp conditions indicating an electroneutral process. The initial rate of calcium uptake was linear up to 15 s. Kinetic analysis of calcium uptake at 7 s showed lower V_{max} and lower K_m in suckling rats compared to adolescent rats. These studies are the first to demonstrate the maturational aspects of calcium entry at the brush border level and are consistent with our previous kinetic studies utilizing whole tissue. (*Pediatr Res* 22: 173–176, 1987)

Abbreviation

BBMV, brush border membrane vesicles

Intestinal calcium transport in the rat has been studied by *in vivo* perfusion techniques (1), everted gut sacs (2), and Ussing chambers (3). These studies provided the overall picture of calcium absorption and has shown that calcium transport occurs by mediated and nonmediated processes (1–3). The mediated process is regulated by 1-25$(OH)_2$ vitamin D_3 the most active polar metabolite of vitamin D (4). The nonmediated process occurs mainly through the paracellular pathway (5). Studies on the developmental aspects of calcium transport using everted gut sacs has shown that the contribution of the nonmediated process to the total process of transport declines with age while the mediated component increases (2). The permeability coefficient of calcium in everted gut sacs was higher in suckling rats compared to adolescent rats. Recently studies using isolated membrane vesicles have provided insight into the process of uptake and exit separately. These membrane vesicles offer the advantage of setting the experimental conditions at will, *i.e.* defining the composition of the media at both sides of the membrane without interference with cell metabolism (6). We have recently validated the use of brush border membrane vesicles for the study of developmental aspects of transport (7, 8).

Using a well-validated technique, we designed the current studies to examine the developmental aspects of calcium transport across the brush border membrane vesicles of the rat intestine.

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Correspondence and reprint requests Fayez K. Ghishan, M.D., Professor of Pediatrics, Vanderbilt University, Medical Center North, D-4130, Nashville, TN 37232.

METHODS

^{45}Ca (14.6-26 mCi/mg) was purchased from New England Nuclear Corp. (Boston, MA). Chemicals were obtained from Sigma Chemical Company (St. Louis, MO). Cellulose nitrate filters, 0.45 μM pore size, were obtained from Sartorius Filters, Inc. (Hayward, CA). All chemicals were of the highest purity available.

BBMV. Sprague-Dawley rats of varying ages (suckling 14–15 days old and adolescent 42–44 days old) were supplied by Sasco Industries (Omaha, NE) suckling rats were purchased with their mothers. Adolescent rats were shipped directly from the supplier. The animals were housed in the animal care facility. Suckling rats were allowed to suckle freely mother's milk, while adolescent rats were fed standard rat chow (Teklad Diets, Madison, WI) *ad libitum* until their sacrifice by cervical dislocation. After sacrifice the entire jejunum was removed, washed with ice cold 0.9% NaCl and everted over a glass rod. The jejunal region extended from the ligament of Treitz to 20 and 50 cm aborally for suckling and adolescent rats, respectively. Brush border membrane vesicles were prepared by sequential precipitation with 0.01 M $MgCl_2$ and differential centrifugation as described previously (7–9). The purity of the vesicle preparation for rats of varying ages has been previously demonstrated (7–9).

Transport measurements. Uptake of radiolabeled calcium was measured by a rapid filtration technique. Typically, incubation was initiated by the addition of 20 μl of vesicle suspension to 60 μl of incubation solution. Experiments were conducted at 25° C unless otherwise stated. After the desired incubation time interval, transport was terminated by abrupt dilution in 1 ml ice cold "stop solution" (100 mM mannitol, 150 mM $MgCl_2$, and 10 mM Hepes/Tris buffer pH 7.4). The reaction mixture was then immediately pipetted onto a prewetted filter (Cellulose Nitrate 0.45 μM pore size, Sartorius Filters, Inc.) and kept under suction. The filter was then rinsed with 5 ml of ice-cold "stop solution" and prepared for scintillation counting. Binding of the radiolabeled calcium to the filter was determined by filtration of incubation media without vesicle protein and was considered as background and substracted from vesicle uptake. Results are expressed as nmol of calcium uptake per mg of vesicle protein. Most experiments were performed in triplicate and repeated at least twice on different days using freshly prepared vesicles. Statistical significance was analyzed by the unpaired Student's t test. K_m and V_{max} were determined according to a computer model for estimation of kinetic parameters (10).

RESULTS

Calcium uptake versus binding. To determine whether calcium uptake represented binding or transport into the intravesicular

space, three experiments were conducted. First, the effect of media osmolality was studied in calcium uptake. To determine whether calcium uptake represents transport into osmotically active spaces or mere binding, BBMV from suckling and adolescent rats were incubated in solutions with various osmolarities and calcium uptake was determined under these conditions. Figure 1 shows that at 7 s calcium uptake into jejunal BBMV from adolescent rats represented uptake into the intravesicular space with small binding component as depicted by the intercept of the relationship of uptake and osmolality (Y = 0.036 + 0.17 ×, ml = 0.96). Similar relationship was obtained with suckling rat BBMV.

Second, the effect of temperature on calcium uptake was determined by comparing uptake at 25 and 0° C. As seen in Figure 2, uptake at 0° C was significantly decreased compared to values at 25° C, suggesting that calcium uptake represents an

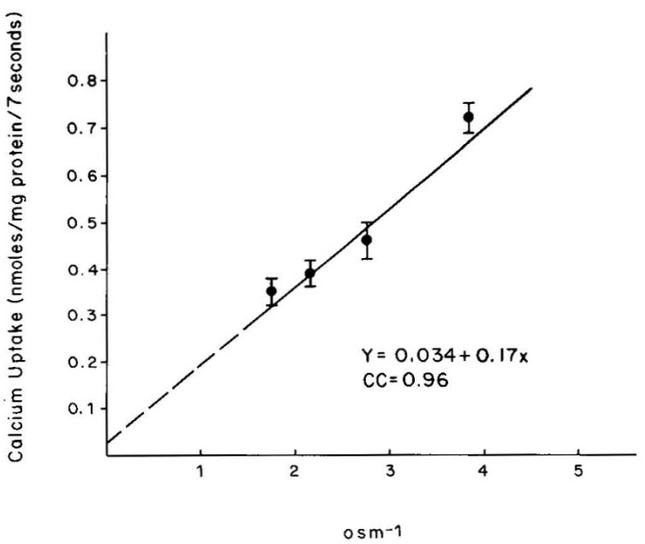

Fig. 1. Effect of media osmolality on calcium uptake. Jejunal BBMV were brought up in 300 mM mannitol and 20 mM Hepes/Tris buffer (pH 7.4). Twenty μl of vesicle preparation was then incubated in incubation media containing 100 mM NaCl and increasing concentrations of mannitol in an effort to increase the media osmolarity. As seen, there is a linear relationship between media 1/osmol and uptake and at infinite osmolality there is minimal binding, indicating that calcium uptake at 7 s represents transport into the intravesicular space.

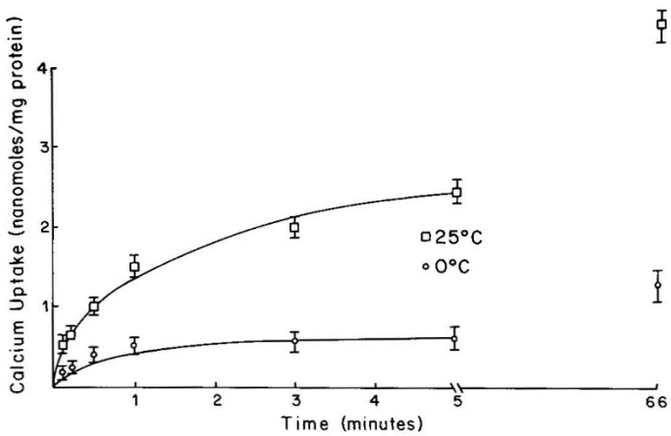

Fig. 2. Effect of temperature on calcium uptake. Calcium uptake was determined in intestinal BBMV from adolescent rats 25 and 0° C. Values represents mean ± SE of (n = 6) determinations. Similar results were also obtained in suckling rats.

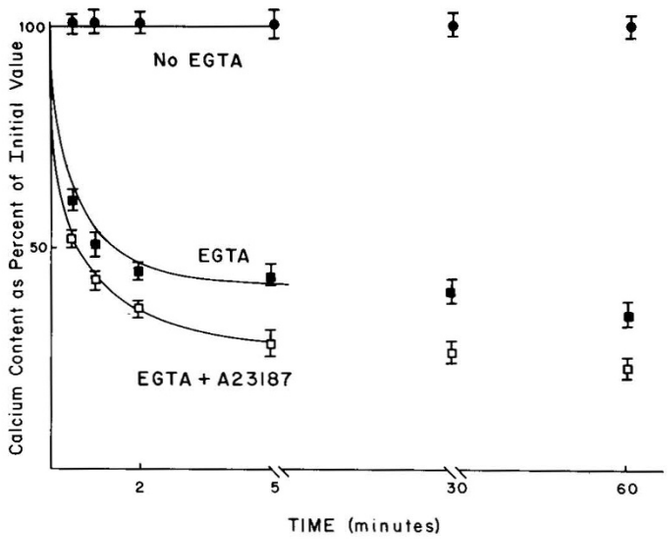

Fig. 3. Effect of calcium ionophore A23187 on calcium efflux. Intestinal BBMV from adolescent rats were loaded into ^{45}Ca for 15 min. Vesicles were then diluted in an incubation media containing calcium ionophore A23187 10 μg/mg protein with or without 1.5 mM EGTA. Samples were obtained at desired time intervals. Values represents mean ± SE of (n = 6) determinations.

energy dependent process rather than mere binding. Third, calcium ionophore A23187 was added at 10 μg/mg protein to BBMV preincubated for 15 min in ^{45}Ca. As seen in Figure 3, calcium efflux occurred rapidly suggesting that calcium was present in the intravesicular space. These observations were seen in both age groups and suggest that calcium transport represented uptake into the intravesicular space.

Calcium uptake with time. Uptake of calcium was determined as a function of time and cationic gradient. BBMV were prepared in a mannitol buffer and incubated in a sodium, potassium or choline containing buffer so that 100 mM cationic gradient was directed from the extravesicular to the intravesicular space. The osmolalities of the incubation solutions remained similar. Figure 4 depicts calcium uptake with time in the presence of sodium, potassium, and choline gradients. Calcium uptake increased with time and shows saturability in both age groups. There was no significant differences in uptake values in the presence of sodium and potassium gradients, while in the presence of choline gradient uptake was significantly decreased in both age groups. Equilibrium values were similar for both age groups. The capability of suckling and adolescent jejunal vesicle preparation for uphill, sodium-coupled transport has been previously shown (7).

Effect of electrical potential. The effect of an imposed electrochemical membrane potential was studied by valinomycin-induced K^+ diffusion potential. Membrane potential was induced by preincubating the vesicles with 50 K gluconate and 200 mM mannitol. The reaction was started by addition of the vesicles to a media containing 10 mM K gluconate, 280 mM mannitol, and valinomycin. Uptake values were compared to those obtained under voltage clamp conditions, *i.e.* vesicles preloaded with 50 mM K gluconate and 200 mM mannitol (K outside = K inside). Because valinomycin mediates the electrogenic movement of K^+ down its concentration gradient, a negative membrane potential is generated. Table 1 shows calcium uptake at 7 s, 0.5, 1, and 5 min under negative membrane potential and voltage clamp conditions in both age groups. There were no significant differences with negative membrane potential indicating an electroneutral process of uptake.

Initial rate uptake. Figure 5 depicts initial rate uptake of calcium. As seen, calcium uptake was linear up to 15 s. The relationship between calcium uptake and time was Y = 0.27 +

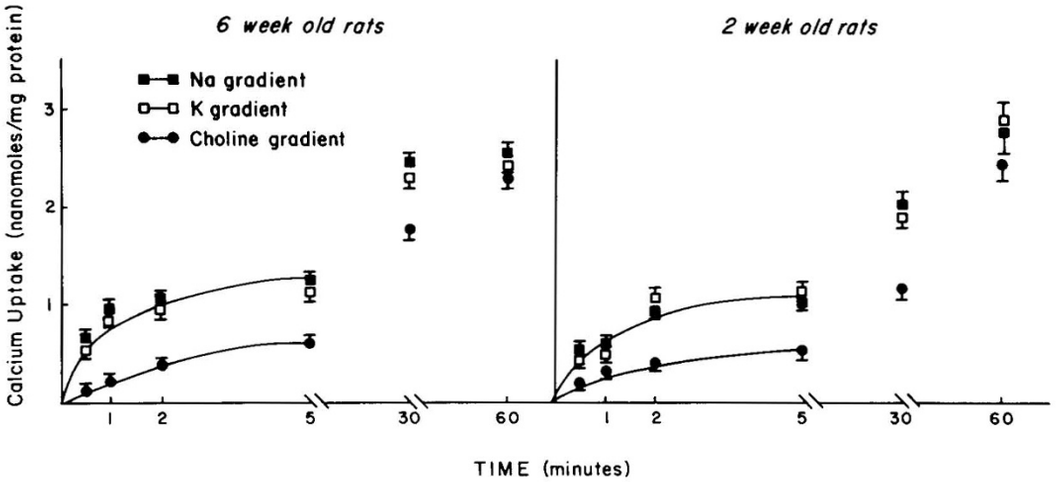

Fig. 4. Effect of cation gradient and time on calcium uptake. BBMV from adolescent and suckling rats were preloaded with 280 mM mannitol and 20 mM Hepes/Tris pH 7.4. Incubations were conducted at 25° C in a medium containing either 100 mM NaCl or 100 mM KCl or 100 mM choline chloride and 100 mM mannitol, 20 mM Hepes/Tris pH 7.4, and 0.1 mM $^{45}CaCl_2$. Values are mean ± SE of (n = 6) determinations.

Table 1. *Effect of membrane potential on calcium uptake by BBMV of adolescent rats**

Time	Negative membrane potential	Voltage clamp
7 s	0.33 ± 00.1	0.32 ± 0.001
30 s	0.92 ± 0.1	0.97 ± 0.1
1 min	1.2 ± 0.2	1.1 ± 0.2
5 min	1.7 ± 0.3	1.8 ± 0.2

* BBMV were preloaded with 50 mM K gluconate, 200 mM mannitol, and 20 mM Hepes/Tris buffer pH 7.4. Incubations were started by the addition of vesicles to a media containing either 10 mM K gluconate, 280 mM mannitol, 20 mM Hepes/Tris buffer pH 7.4, 0.1 mM $^{45}CaCl_2$, and 10 μg protein of valinomycin "voltage clamp" conditions. Values are mean ± SE of (n = 6) determinations. Similar results were obtained with suckling rat brush border membrane preparations.

0.037 × with correlation coefficient = 0.985. The positive intercept represents rapid initial binding component (11).

Kinetics of calcium uptake. Kinetics of calcium uptake was determined at 7 s well within the linear phase of uptake. Figure 6 depicts total uptake of calcium plotted against calcium concentration. K_m and V_{max} for calcium transport in both suckling and adolescent rats was determined. As seen, both K_m and V_{max} were lower in the suckling period compared to corresponding kinetics in adolescent rats ($p < 0.01$).

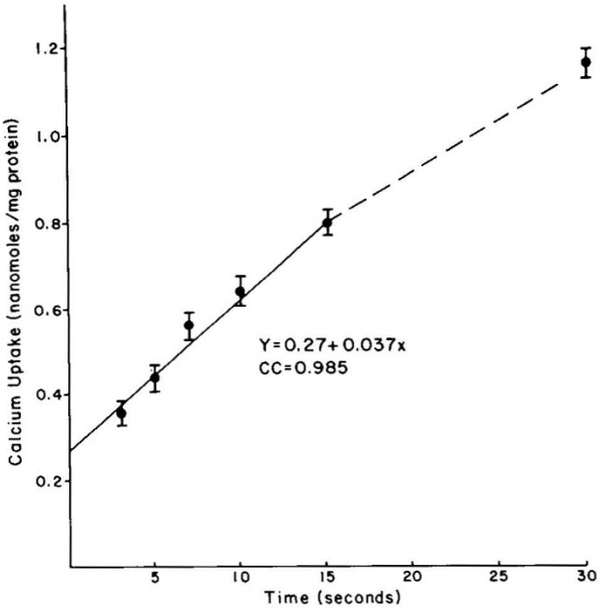

Fig. 5. Initial rate of uptake of calcium. BBMV were brought up in mannitol buffer pH 7.4. Calcium concentration was 0.1 mM. Calcium uptake was determined up to 30 s. The uptake appears linear up to 15 s. Values are mean ± SE of (n = 6) determinations.

DISCUSSION

Calcium transport across the intestinal mucosa occurs via transcellular and paracellular pathways (12). In the classic *in vivo* perfusion studies and *in vitro* gut sacs, both pathways are studied together. Moreover, transepithelial calcium transport represents entry at the brush border membrane and transcytosolic movement and exit at the basolateral membrane. Recently, BBMV have been utilized to define events at the entering step of transport without the interference of cellular energy. Our studies utilized a well validated technique to investigate the maturational changes in calcium uptake at the brush border level.

Previous studies in our laboratory have validated the use of BBMV from the jejunum and ileum of rats during maturation (7–9). These studies included morphological, biochemical, and functional criteria which validated the use of these vesicles for transport studies during maturation (7–9).

In order to define whether calcium uptake represent binding or transport into the vesicular space, three studies were conducted. First an osmolality experiment was conducted. In this setting, calcium uptake was determined at 7 s in a media with increasing osmolality. Calcium binding at this early time point represented mostly transport into the intravesicular space with a small binding component. The relationship between calcium uptake and 1/osmol is expressed as Y = 0.036 + 0.17X with correlation coefficient = 0.96. Thus at isotonicity only 15% of uptake represented binding. In the second experiment calcium uptake was determined at 25 and 0° C. The decrease in calcium transport at 0° C compared to 25° C is consistent with the concept that a major component of calcium uptake represents transport into the intravesicular space rather than binding. Carrier-mediated transport phenomena in general are temperature dependent (13). In the third experiment, calcium ionophore A23187 was added to vesicles preloaded with ^{45}Ca. The presence of A23187 enhanced calcium release compared to EGTA indicating the presence of calcium in the intravesicular space. These findings

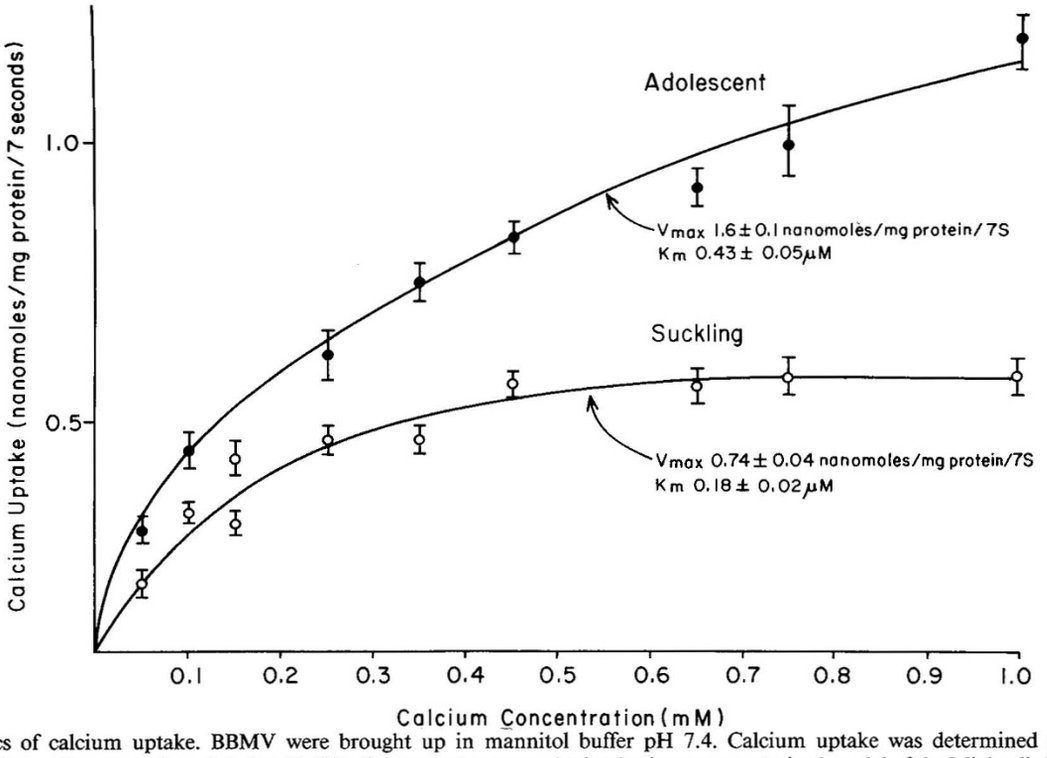

Fig. 6. Kinetics of calcium uptake. BBMV were brought up in mannitol buffer pH 7.4. Calcium uptake was determined at 7 s. Calcium concentration in the media ranged from 0.1–1 mM. Kinetic parameters were obtained using a computerized model of the Michaelis-Menten kinetics.

are similar to those of Schedl and Wilson (14). To determine the driving force for calcium uptake by BBMV, uptake studies were done in the presence and absence of sodium gradient. As seen in Figure 4 calcium uptake was similar with Na^+ or K^+ gradient indicating that sodium gradient is not the driving force for calcium uptake. Choline gradient depressed calcium uptake as compared to Na^+ or K^+ gradient conditions. Calcium uptake, however, showed saturability with time indicating a facilitated diffusion or a carrier-mediated process. These findings are similar to that of Miller and Bronner (15) in which calcium uptake was not sodium dependent and decreased in the presence of choline. An imposed electrical potential as induced by the ionophore valinomycin had no effect on calcium uptake indicating an electroneutral process. A similar process was found with phosphate uptake across the brush border membranes (9). In contrast, D-glucose uptake in this setting was significantly enhanced compared to Na^+ gradient conditions alone suggesting an electrogenic process (7). Miller and Bronner (15) found slight increase in calcium uptake at 15 min under negative membrane potential but no increase at 0.5 min. The kinetics of calcium uptake was determined after the demonstration of the linearity of uptake up to 15 s. Therefore kinetics of calcium uptake were determined at 7 s well within the linearity of uptake. K_m and V_{max} were lower in suckling rats compared with those of adolescent rats, indicating greater affinity but lower capacity in the infant rats compared to adolescent rats. These kinetic parameters are similar to our whole tissue studies in which the V_{max} and K_m of the mediated (*i.e.* transcellular component) was small in suckling rats and increased with age. Thus, events at the brush border membrane reflects observations with whole tissue.

Our previous *in vivo* (1) and *in vitro* gut sac (2) studies indicated that the intestinal calcium transport in the suckling period occurs mainly by a passive process through the paracellular pathway. The transcellular movement was small and increased with age. This study using BBMV representing transcellular movement, agrees with our previous studies, indicating an increase in transcellular calcium transport with advancing age.

In summary, our data suggest that calcium uptake into brush border membranes represents uptake into the intravesicular space. This process is electroneutral, saturable, and not sodium dependent. The kinetic parameters of this process show maturational changes which evolve with age, and are similar to events seen with whole tissue.

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