Apparent Increase in Carrier Affinity for Intestinal Calcium Transport following Dietary Calcium Restriction*

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SUMMARY

Rats fed a diet lacking calcium adapt to this deprivation by increased active transport of this ion across the intestine. When the characteristics of the transport system were analyzed in vitro by use of saturation kinetics, increased net absorptive calcium transport appeared to be the result of an increased affinity (decrease in $K_t$ from 1.12 to 0.59 mM) rather than an increase in the capacity or $V_{\text{max}}$ of the transport process. Adaptation through substrate induction in yeast results in an analogous increase in carrier affinity for sugar transport by a linkage to metabolic energy. Such a mechanism could account for the lowered dietary calcium effect, however the authors suggest that rather than an actual change in carrier affinity a second molecule (such as intestinal calcium-binding protein) could also alter carrier-substrate equilibria and produce a decrease in the observed $K_t$ if access to the carrier were rate limiting at low substrate concentrations and this second molecule increased access of substrate. This model is consistent with the changes in calcium transport system characteristics and calcium-binding protein levels under conditions of restricted dietary calcium levels.

Adaptation of cellular membrane transport in most microorganisms occurs by induction in the presence of the substrate to be transported; but in a few cases, high substrate levels repress rather than induce transport (1). In bacteria, the protein synthesis which results from either induction or derepression increases the total number of "carrier" molecules (2). However in yeast, several transport processes which appear to be constitutive facilitated diffusion systems, adapt as a result of substrate induction by a coupling of metabolic energy to these carrier systems which then become capable of active transport (3, 4). Mechanisms of adaptation in metazoans have not been as well defined. Perhaps the best studied example of mammalian adaptation to low substrate levels, which is not under known hormonal control (5, 6), is an animal's ability to increase its capacity for calcium absorption when dietary calcium is restricted (5, 6).

Recently we reported that a low calcium diet increased the active transport of calcium from the mucosal to the serosal side of the intestinal wall ($J_{\text{MS}}$); while movement in the opposite direction ($J_{\text{SM}}$) was unaffected (7). This enhancement of $J_{\text{MS}}$ is blocked by puromycin (8), implicating protein synthesis in the low calcium effect on transport. We were unable to determine whether the LCD-mediated increase in $J_{\text{MS}}$ occurred as an increase in total capacity ($V_{\text{max}}$) of the active transport process, an increase in the affinity of the proposed carrier, reflected by a decrease in $K_t$ (the apparent Michaelis constant), or by a combination of changes. The present study was designed to investigate how the increase in calcium transport resulting from low calcium diet occurs by examining the kinetic parameters of the process across rat duodena in vitro.

METHODS

Male Holtzman rats weighing 140 to 200 g were fed Purina Laboratory Chow for at least 1 week prior to experimentation. In these studies on the effects of lowered dietary calcium rates were fed the Harvard low calcium diet (9) for 1 week prior to killing. Animals were fasted overnight, killed by concussion, and the uppermost 2 cm of duodenum excised, cut along the mesenteric attachment, rinsed in 0.9% sodium chloride solution and mounted in vitro as the partition between two half-chambers. The tissue (area = 0.49 cm²) was bathed in a modified Krebs-Henseleit-Ringer's solution (bicarbonate buffer) containing 0.125 to 10 mM CaCl₂ as indicated, no inorganic phosphate, and 11 mM glucose as a metabolic substrate. The solutions were aerated with 95% O₂-5% CO₂ and maintained at 37°C. Each compartment contained 15 ml of fluid at pH 7.4 and volumes remained equal during the experimental period.

Calcium fluxes were monitored with $^{48}$Ca as previously described (7). Flux values for individual experiments are given as the mean of the steady state flux measurements (see Reference 7) (3 to 5 steady state samplings per experiment). This calculated mean represents a $n = 1$. The transmural potential difference was measured (7) and junction potentials were always

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1 The abbreviations used are: LCD, low calcium diet; CaBP, calcium-binding protein.
less than 0.2 millivolts. The potential difference was not short-circuited.

Statistical differences between flux rates were determined with Student t test. A program for least squares fit was used to obtain a description of the lines in Figs. 2 and 3. Statistical differences between the slopes of the linear transformations of the Michaelis-Menten equation were determined by the method described by Cleland (10).

**EXPERIMENTAL DESIGN**

Intestinal calcium absorption is an extremely labile process which responds to the metabolic calcium requirements of the animal; at least in the presence of adequate vitamin D. Growth, pregnancy, and dietary calcium deprivation all increase the capacity of the absorptive process or processes (5, 11). In this study, small differences in age and sex as yet unaccounted for intergroup variance (happening between animals of the same strain, sex, and age) when observed at different times produced significant changes in Jms. An extreme example of this unexplained intergroup variance is given in Table I. The Jms for one low calcium diet group is approximately the same as the normal flux in the second group. If these values are compared, then the erroneous conclusion would be that there was no low calcium effect. On the other hand, if we compare the normal Jms from Group 1 and the LCD Jms from Group 2, there is an apparent enhancement of the low calcium effect. If, however, the intragroup controls are used in making comparisons, the magnitude of the low calcium effect is equivalent in both cases (Table I). To avoid the possibility of drawing misleading conclusions from this type of data we compared internally controlled experimental groups. Because a large number of animals was required in each group as a result of the 3 × 4 experimental design, these experiments took 10 days to complete; therefore, intragroup variance because of small age differences became unavoidable and age-dependent decreases in transport capacity were produced (resultant weight range, 140 to 200 g) (see Fig. 1).

To minimize the effect of age differences, normal and LCD groups were studied on consecutive days at each concentration of calcium with normal groups being done first. Furthermore, experiments were performed in order of decreasing calcium concentration to eliminate the possibility that saturation phenomena at high calcium concentrations could be the result of age-related differences in transport rates.

**TABLE I**

**Intergroup variability of calcium transport**

Steady state transmural calcium fluxes expressed as nanomoles cm⁻² 10 min⁻¹ were studied at a [Ca²⁺] = 1.25 mM in rats of approximately the same age (140 to 160 g). The results represent an extreme example of intergroup variability between groups of rats on normal and low calcium diets studied at different periods. While baselines differ for the normal groups, the magnitude of the LCD effect is the same.

<table>
<thead>
<tr>
<th>Calcium flux</th>
<th>n Group 1</th>
<th>±</th>
<th>n Group 2</th>
<th>±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jms Norm</td>
<td>5</td>
<td>17.1 ± 0.6 a</td>
<td>10</td>
<td>28.2 ± 2.1</td>
</tr>
<tr>
<td>Jms LCD</td>
<td>4</td>
<td>29.9 ± 0.1 b</td>
<td>9</td>
<td>40.3 ± 3.2 c</td>
</tr>
<tr>
<td>Jsm</td>
<td>7</td>
<td>7.6 ± 0.7</td>
<td>7</td>
<td>6.7 ± 1.0</td>
</tr>
<tr>
<td>ΔLCD a</td>
<td>12.8</td>
<td></td>
<td>12.1</td>
<td></td>
</tr>
</tbody>
</table>

a S.E.

b Jms LCD > Norm p < 0.001.

c Jsm LCD > Norm p < 0.01.

d ΔLCD = Jms LCD − Jsm Norm.

**TABLE II**

**Changes of transmural calcium flux with calcium concentration**

Steady state transmural calcium fluxes expressed in nanomoles cm⁻² 10 min⁻¹ were studied over a range of [Ca²⁺] in animals on normal and low calcium diets. Active calcium transport was found at all [Ca²⁺] (Jns/JM > 0.67) and LCD increased mucosal to serosal flux at the lower [Ca²⁺].

<table>
<thead>
<tr>
<th>Calcium flux</th>
<th>0.125 mM</th>
<th>1.25 mM</th>
<th>5 mM</th>
<th>10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jms Normal</td>
<td>4</td>
<td>3.1 ± 0.4 a</td>
<td>10</td>
<td>32.7 ± 2.0</td>
</tr>
<tr>
<td>Jms LCD</td>
<td>5</td>
<td>4.7 ± 0.5 b</td>
<td>9</td>
<td>44.7 ± 3.3 c</td>
</tr>
<tr>
<td>Jsm</td>
<td>7</td>
<td>0.7 ± 0.06</td>
<td>7</td>
<td>6.7 ± 1.0</td>
</tr>
</tbody>
</table>

a S.E.

b Jms LCD [Ca²⁺] = 0.125 mM > Jms Normal, p < 0.02.

c Jms LCD [Ca²⁺] = 1.25 mM > Jms Normal, p < 0.01.

d Jsm LCD [Ca²⁺] = 5 mM > Jsm Normal, p < 0.05.
dissolved decreases in transport ability. This experimental design is therefore biased against the indication of low calcium diet effect or saturation phenomena as results of artifact caused by age differences or intergroup variance.

RESULTS

Bidirectional transmural fluxes of calcium across rat duodena were measured over a range of calcium concentration (0.125-10 mM) in animals fed normal and low calcium diets. The transmural potential difference was 4 to 6 millivolts with the serosal side of the tissue positive relative to the mucosal (potential difference was unchanged by LCD and [Ca^{2+}]) (7). Our previous finding that JSM was unaffected by LCD (7) was confirmed and JSM values from both experimental groups are pooled. We have also previously shown that net movement of calcium from the mucosal to serosal side of the tissue against this potential difference is compatible with thermodynamic criteria for active transport (7). The passive flux ratio given by the Ussing equation (12) JMS/JSM = 0.67. In the current study, active transport of calcium in inorganic phosphate-free media was observed in both normal and LCD animals at all concentrations of calcium investigated (Table II). Low calcium diet increased JMS at calcium concentrations of 0.125, 1.25, and 5 mM (Table II). At 10 mM [Ca^{2+}], however, LCD and normal JMS were identical (p > 0.995, Table II). Serosal to mucosal flux changed linearly over this concentration range (r = 0.98, Fig. 2); consistent with passive diffusion or a carrier-mediated process with a low substrate affinity. Both LCD and normal JMS approached a plateau between 3 and 10 mM [Ca^{2+}], but the curves were not ideal rectilinear hyperbolas. As we previously suggested (7), these saturation phenomena are consistent with a carrier-mediated active transport process. Since there is a diffusional calcium flux (presently quantitatively indeterminable) in addition to the carrier-mediated flux, the JMS curve can be approximated by the Michaelis-Menten equation plus a maximal estimate of diffusion with the Ussing flux ratio.

$$J_{MS} = [S] \times \frac{V_{max}}{K_t + [S]} + J_{SM} \times e^{-\frac{zF\Delta \Phi}{RT}}$$  (1)

Observed JMS was corrected for diffusion by subtracting 0.67 \times JSM (7) and is referred to as "JMS corrected."

In accordance with the suggestions of Dowd and Riggs (13), the linear transformation \( J = \frac{V_{max} - K_t}{J/[S]} \) was used to analyze the data instead of the method of Lineweaver and Burk.

A close coincidence of \( K_t \) and \( V_{max} \) was found when comparing uncorrected JMS extrapolated from the curves in Fig. 2 to the values derived from this transformation (Fig. 3) (observed LCD

![Figure 2](http://www.jbc.org/)

**FIG. 2.** Steady state calcium flux versus [Ca^{2+}]. LCD, low calcium diet. Normal and LCD \( K_t \) are extrapolated from half the calculated maximal velocity.

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Linear transformation of the Michaelis-Menten equation. \( V_{max} \) = intercept at y axis; Slope \( b = -K_t \). LCD, low calcium diet; JMS corrected = JMS observed - 0.67 JSM. Observed normal \( K_t > \) observed LCD \( K_t, P < 0.001; \) corrected normal \( K_t > \) corrected LCD \( K_t, P < 0.001. \)

**TABLE III**

Similarities between normal and LCD transmural calcium fluxes at high calcium concentrations

The data represent steady state calcium fluxes expressed in nanomoles cm^{-2} 10 min^{-1} from animals on normal and low calcium diets studied at relatively high [Ca^{2+}]. The calcium fluxes are expressed as observed fluxes (e.g. LCD JMS) and also as corrected fluxes when the mucosal to serosal flux had been maximally corrected for diffusion with an estimate of diffusion from the Ussing equation (corrected JMS = observed JMS - 0.67 \times JSM). At all three calcium concentrations, LCD JMS did not exceed normal JMS, indicating that the maximal transport capacity is the same for both groups.

<table>
<thead>
<tr>
<th>[Ca^{2+}]</th>
<th>Calcium flux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal JMS</td>
</tr>
<tr>
<td>mM</td>
<td>n</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>12.4</td>
<td>3</td>
</tr>
</tbody>
</table>

* S.E.
"Kt = 1.70 mM, calculated LCD Kt = 1.88 ± 0.07 S.E.; observed normal Kt = 3.05 mM, calculated normal Kt = 3.03 ± 0.08).

The V_max for J_M was not increased by LCD with either observed or corrected data (Fig. 3). In other experiments, J_M was found to be the same in both normal and LCD animals at several high calcium concentrations (Table III).

Since V_max was unchanged and J_M for LCD animals was greater than normal J_M at low calcium concentrations, (with the analogy to enzyme kinetics) K_t should have been decreased by LCD. This was indeed the case and the normal K_t was almost twice LCD K_t for the uncorrected data (normal K_t = 3.03 ± 0.08 mM S.E., n = 25; LCD K_t = 1.88 ± 0.04/, n = 21; p < 0.001) as well as for “J_M corrected” (normal K_t = 1.12 ± 0.06 mM S.E.; LCD K_t = 0.50 ± 0.06; p < 0.001).

The contribution of processes other than the proposed carrier system cannot be accurately estimated, and therefore the corrected and uncorrected constants reflect the extremes within which each “true K_t” lies. Since LCD does not change J_M (7), the diffusional characteristics of the tissue appear unchanged and the difference between normal and LCD K_t is therefore constant within the observed ranges.

**DISCUSSION**

Enhancement of J_M by low calcium diet appears to occur because of an increase in the affinity of the transport system (indicated by the decrease in K_t) rather than by an increase in total capacity. This interpretation of the data depends upon the appropriateness of the analogy to enzyme-substrate interactions; with the principal assumption being that the observed K_t reflects carrier-substrate association and dissociation. In the subsequent discussion, we will show that use of the Michaelis-Menten kinetic model may indeed be an oversimplification.

The kinetic constants for “corrected J_M” are quite reproducible. The K_t and V_max in these experiments for low calcium diet J_M agree well with those we previously reported (which when calculated from the J/[S] versus J transformation are, LCD V_max = 55.3 nmoles-cm⁻²·10 min⁻¹; K_t = 1.00 mM ± 0.09 S.E., n = 14) (7). The V_max for this duodenal transport process therefore appears to be 80 to 55 nmoles of calcium per cm²·10 min, regardless of the level of dietary calcium and over a range of ages in growing animals. The K_t is about 1 mM and apparently decreases by almost one-half when dietary calcium deprivation increases the animal’s need for calcium. Whether the same kinetic constants would be found in adult animals or in animals that are vitamin D deficient remains to be determined.

Several different mechanisms have been described by which the K_t for transport processes can be decreased without a change in V_max and they must be considered in relation to the low calcium effect on K_t. First, is the possibility of a hormonal interaction such as that described for the effect of insulin on amino acid uptake by diaphragm (14). The low calcium effect however, seems to be independent of any known hormonal regulation (5, 8). In addition, its time course is much slower than the insulin-mediated change and protein synthesis is apparently directly involved (8).

Another manner in which a K_t change might occur is by a mechanism similar to the sodium dependence of cotransport systems for sugar and amino acids. Since the composition of solutions were constant in these experiments (except for [Ca²⁺]) a decreased K_t from a second substrate interacting with the carrier could not have occurred in this way unless low calcium diet changed intracellular ionic gradients.

Perhaps a change in transport kinetics more analogous to the LCD effect is the substrate-induced active transport of sugars in the yeast, *Saccharomyces cerevisiae* (since the mechanism of the low calcium effect is not known, both substrate derepression and a hormone-like induction must be considered). This yeast has a constitutive maltose facilitated diffusion system which, when induced in maltose-containing media, acquires the ability to actively transport maltose (3). This change occurs through a decrease in K_t (3, 15) without a change in V_max (15). This change in K_t is thought to occur by an energy-linked increase in the affinity of the influx carrier while the affinity of the efflux carrier is unchanged (3). Conversely, in several bacterial systems it has been suggested that the apparent increase in carrier affinity occurs through an energy-linked decrease in affinity of the efflux carrier (16). Either of these mechanisms which change net transmembrane flux by unidirectionally affecting carrier affinity could account for the low calcium effect on K_t.

However, if the vitamin D-dependent calcium-binding protein is assumed to have a role in calcium transport, other means of K_t change must be considered. When adequate dietary vitamin D is available, low and high calcium diets result in proportionate increases and decreases in CaBP levels and in calcium active transport (17, 18). This correlation of flux to CaBP together with the vitamin D dependence of both transport and CaBP strongly suggests a function of CaBP in the calcium transport process. We have made two observations that indicate that CaBP is not the carrier whose kinetics we have observed. First, since CaBP levels (assayed by increased supernatant binding) and calcium transport are nearly doubled by LCD (18), if CaBP were the carrier molecule in the rate-limiting process, the greatly increased level of CaBP would represent a similar increase in carrier sites which would require that the V_max increase proportionally. The second finding is that the apparent affinity of the transport system for calcium (K_t) is about 1 mM while the dissociation constant (K_D) of CaBP at the same ion strength (γ/2 = 0.16) is 4 μM (19). The proposed carrier proteins isolated from bacteria have values for K_t and K_D that are much more similar (2). This apparent difference in affinity between the calcium active transport system and CaBP is remarkably similar to the recent finding of Eichholz, Howell, and Crane (20), that the K_D (called K_m by these authors) for binding of D-glucose to a subtraction of intestinal brush border is 2 μM while the K_t (also called K_m (20)) for active sugar transport is 1 μM. They tentatively suggest that this brush border binding may serve a concentrative or scavenging role rather than functioning as part of the active transport process.

The cellular localization of CaBP is unclear. Immunofluorescent antibody studies suggest that CaBP is in goblet cells and in the vicinity of the brush border (glycocalyx) (18). However, CaBP is recovered in the soluble fraction of homogenates (21) and more recent studies by Bronner’s group suggest that the locus is intracellular (8). Until a definite localization of CaBP is achieved, both a glycocalyx, membrane site, or both, and an intracellular location must be considered.

A model can be proposed which is consistent with observed changes in K_t and CaBP levels without changing V_max. It would require that at low external concentrations of calcium, access to the membrane “pump” (serosal, mucosal, or lateral)
was rate limiting and that the number of transport sites was unchanged. A process that would increase availability to the active site at low [Ca^{2+}] would decrease the observed $K_i$ without increasing $V_{\text{max}}$. Calcium-binding protein could function either in the glycoalyx or intracellularly to bind and then make calcium available to the active process. In an intracellular locus CaBP might also function to increase net flux into the cell by binding entering calcium thereby decreasing loss because of efflux as well as maintaining an activity gradient into the cell. For both of the above models, the change in $K_i$ would not involve changes in the carrier affinity per se as is implied by the apparent Michaelis constant, but would occur through a second molecule limiting the interaction of substrate with the carrier.

Since calcium transport is active, an influx-efflux carrier affinity difference exists. In the models considered, low calcium diet could further increase this affinity difference either by a means such as an energy link that would alter the carrier affinity directly or through the action of another molecule in the transport process (CaBP) that would regulate the interaction of substrate with the carrier.

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