Phosphate-independent, carrier-mediated active transport of calcium by rat intestine

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Walling, Marlin W., and S. S. Rothman. Phosphate-independent, carrier-mediated active transport of calcium by rat intestine. Am. J. Physiol. 217(4) : 1144-1149. 1969.—Steady-state calcium fluxes were measured across segments of proximal rat duodenum mounted in vitro across two half-chambers. Active transport of calcium was observed in growing animals in the absence of concentration gradients and against a mean transmural potential of 5.2 mv (mean MS flux/SM flux = 2.2). Net calcium transport was greatly diminished in adult animals. Contrary to a previous report, we found that active calcium transport was not inhibited when inorganic phosphate was absent from the bathing solution. When fluxes were studied over a 100-fold range of calcium concentrations (0.125-12.5 mM), MS flux appeared to saturate while SM flux increased proportionally with calcium concentration. Calcium MS flux was approximately doubled (mean MS flux/SM flux = 4.2) in rats fed low-calcium diet (LCD), while SM flux was unchanged. This LCD effect apparently represents an increase in the capacity of the active transport process. The saturation, LCD enhancement, and vitamin D dependence of the MS flux process suggest carrier-mediated calcium transport.

low-calcium diet; dietary adaptation; saturation kinetics; ion transport; duodenum; calcium absorption

THE INADEQUACIES of data supporting an active transport model for calcium absorption (11-13, 22) were pointed out by Helbock et al. (4). They studied the movement of calcium across rat duodenum using in vivo and in vitro perfused intestinal loops and a modification of the in vitro system of Ussing and Zerahn (18). In the absence of inorganic phosphate (Pi) and with the transmural potential (Pi) short circuited, they found no active transport of calcium (MS flux/SM flux = 0.97; [Ca++] , 1(10^-2 M). (MS flux refers to movement from the fluid bathing the mucosal surface of the intestine to the fluid bathing the serosal surface; SM flux is movement in the opposite direction). Calcium fluxes were also studied over a 1,000-fold range of calcium concentrations (from 1(10^-4) to 1(10^-3) M), and changes in calcium transport were linear which suggested to them that either passive diffusion or a carrier-mediated system well below saturation was involved. Phosphate fluxes were studied under short-circuited conditions and active transport of Pi was observed (MS flux/SM flux = 1.54). Furthermore, when Pi was added to the bathing media ([Pi], 3.7 mM; [Ca++] , 1(10^-8) M), the calcium flux ratio was increased to 1.56, i.e., apparent active transport of calcium. Phosphate also greatly increased calcium absorption from in vivo perfused gut loops and a variety of chelating agents similarly increased calcium MS flux both in vivo and in vitro. Primarily on the basis of these results, Helbock and his co-workers (4) concluded that there was no active transport of calcium per se across the duodenum, nor was there any evidence for a facilitated diffusion system. They hypothesized that apparent active calcium transport was the result of calcium movement linked by some means to Pi, active transport.

The present study presents evidence that calcium transport by rat duodenum; 1) is active, 2) occurs in the absence of Pi, 3) is carrier mediated, 4) is unidirectionally enhanced when animals are placed on a low-calcium diet (LCD).

METHODS

Studies were conducted in vitro using a modification of the apparatus of Ussing and Zerahn (18). Male Holtzman rats weighing 150-200 g and fasted overnight were sacrificed by concussion. The uppermost 2 cm of duodenum was excised, cut along the mesentric attachment, rinsed in isotonic saline and mounted in vitro as the partition between two round half-chambers. The mounted tissue (area = 0.49 cm²) was bathed in a modified Krebs-Henseleit-Ringer solution (bicarbonate buffer, pH 7.4) containing 1.25 mM CaCl₂ unless otherwise indicated, either 0 or 7.4 mM Pi as indicated, and 11 mM glucose as a metabolic substrate. Each compartment contained 15 ml of fluid. The solutions were aerated with 95% O₂-5% CO₂. Bathing solutions were circulated through a water bath maintained at 37 C using a photoflood light as a heat source and a thermoregulator with a thermal sensor probe. Calcium fluxes were monitored using 45CaCl₂ (New England Nuclear Corp.) as a tracer with approximately 1.5(10⁷) counts/min being present in the compartment to which the isotope was added. The resulting specific activity was about 1 µc/µmole ([Ca]1⁴⁺, 1.25 mm). One-milliliter samples were taken at 10-min intervals from the initially “cold” compartment and immediately replaced by an equal aliquot of cold solution. The samples were plated on aluminum planchets, dried at constant temperature and counted in a thin window gas-flow Geiger counter with a maximum of 3% as the standard error of counting. Self-absorption corrections were not necessary.

The transmural potential difference was monitored via Krebs-Henseleit-Ringer salt-agar bridges and paired calomel half-cells connected to a millivolt potentiometer (Kcitllley 400B). Junction potentials were always less than 0.2 mv. The PD was not short circuited.
Animals were fed Purina laboratory chow for at least 1 week prior to experimentation. In those studies on the effects of lowered dietary calcium, rats were fed the Harvard low-calcium diet (3) for 1 week prior to sacrifice. The rachitogenic diet was LCD with normal calcium salts added and vitamin D omitted. Animals were kept in an environment free from ultraviolet light and severe growth retardation was used as evidence of vitamin D deficiency.

Flux values for individual experiments are given as the mean of the steady-state flux measurements (3-5 steady-state samplings/experiment). This calculated mean represents an N = 1.

RESULTS

Rat small intestine is electrically polarized so that the serosal surface is positively charged relative to the mucosal. The mean transmural PD was 5.2 mv ± 0.4 SEM when the tissue was first mounted in vitro and gradually fell to 3.8 mv ± 0.1 SEM after 1.5 hr incubation. The electrical potential across the rat small bowel was used to monitor the viability of the preparations because this potential appears to be generated by the metabolically dependent active transport of sodium ion (4, 14).

The MS and SM fluxes across the whole intestinal wall are the resultants of individual efflux and influx events occurring at both the luminal and contraluminal membranes of the mucosal cells as well as across a serosal barrier (e.g., connective tissue and muscle). In a group of experiments, the intestinal epithelium and underlying tissue were scraped from the serosal musculature. This remaining tissue, as expected, behaved as a simple diffusional barrier with a permeability about twice that of the intact tissue. This tissue was therefore not rate limiting as far as diffusion across the whole intestinal wall was concerned.

Active transport of calcium. In normal animals a large net MS flux of calcium was observed (MS flux = 17.1 ± 0.6 SEM μmols/cm² × 10 min, N = 5; SM flux = 7.9 ± 2.0, N = 4; P < 0.005) (Fig. 1). Since the net flux in the steady state was electrochemically uphill, these results demonstrate active transport of calcium. The passive flux ratio predicted by Ussing’s equation (17) at a PD of 5.2 mv was much lower (0.68) than the ratio observed for mean MS flux/SM flux in normal animals (2.2), again indicating active transport of calcium. The metabolic dependence of both net calcium absorption (13) and tissue uptake of 45Ca by slices of rat duodenum (12) has been previously demonstrated in vitro and we have confirmed this relationship in other experiments.

Intestinal calcium absorption is dependent on an adequate supply of vitamin D (3, 8, 13). If the observed asymmetric flux ratios were produced by sources other than the active transport of calcium (e.g., solvent drag), then an equivalent asymmetry would be expected in vitamin D-deficient animals if other intestinal transport processes are unaffected by this deficiency. Furthermore, vitamin D supplementation would not be expected to alter this relationship. The effect of vitamin D was tested in the present system using 50-to-70 g animals placed on a vitamin D-free diet for 10 days.

In the vitamin D-deficient animals, MS flux was no different than SM flux (−vitamin D MS flux = 24.3 ± 4.0 SEM μmols/cm² × 10 min, N = 4; SM flux = 21.1 ± 3.3, N = 4). The PD was 10.2 ± 0.4 SEM mv in these very young animals, indicating that sodium transport and any resulting water movement were not depressed. Tissue from these animals was considerably thinner than tissue from older animals (mean weight of the 0.49-cm² piece of tissue mounted across the chamber for 50- to 70-g rats = 19.5 ± 1.4 SEM mg wt weight, N = 11; for 150- to 200-g rats = 30.0 ± 0.4, N = 11) and this difference may result in a decreased diffusional barrier which would account for the higher bidirectional flux rates. When the rachitogenic diet was supplemented with vitamin D (calciferol) for 48 hr, MS flux more than doubled (vitamin D MS flux = 60.2 ± 10.2 SEM μmols/cm² × 10 min, N = 3; + vitamin D MS flux > − vitamin D MS flux, P < 0.02), while the PD was unaffected. The effect of vitamin D on SM flux was not measured so that bidirectional effects of vitamin D cannot be evaluated.

Transport enhancement by lowered dietary calcium. The preceding results show that calcium transport is directional in segments of duodenum from growing normal animals bathed in a P_i-containing medium. Dietary calcium deprivation is known to enhance an animal’s ability to absorb calcium (3, 6, 8). Studies using everted gut sacs have demonstrated that LCD increases serosal to mucosal fluid 45Ca ratios relative to controls (6). However, these studies did not determine which flux processes were altered, i.e., decreased SM flux, increased MS flux, or some combination of changes. In our experiments, unidirectional fluxes were measured in order to determine which of these changes were occurring. In LCD rats the mean calcium MS flux was greatly increased over controls (P < 0.001) while the SM flux was unchanged (Table 1). The PD was unaffected by LCD (PD = 5.3 ± 0.2 SEM mv, N = 38). The LCD enhancement was therefore on the MS flux or active component of the calcium transport system. The mean MS flux:SM flux ratios were approximately doubled (2.2 normal vs. 4.2 LCD). This effect may represent an induction of active transport and will be discussed later in more detail.

Inorganic phosphate dependence of calcium transport. Since it has recently been suggested that P_i transport is requisite for the net movement of calcium (4, 7), calcium fluxes in normal and LCD animals were also studied in a P_i-free medium. In the absence of P_i, MS flux was not depressed and SM flux was unchanged (Table 1). In LCD animals MS flux...
was actually increased in the absence of Pi ($P < 0.05$) (Table 1). The removal of Pi did not alter PD in either normal or LCD animals. Therefore, active calcium transport in this system at the concentrations studied was not dependent on the presence of phosphate in the bathing solution.

Effect of age on calcium transport. Bidirectional calcium fluxes were also studied in adult (330- to 400-g) male Holtzman rats at a $[\text{Ca}^{2+}]$ of 2.5 mM. In the absence of Pi there was no net calcium transport (MS flux ($N = 4$) = $18.2 \pm 4.8$ SEM mmol/cm$^2 \times 10$ min; SM flux ($N = 3$) = $15.2 \pm 4.1$; $P > 0.6$). The mean MS flux to SM flux ratio was 1.19. Essentially the same ratio was observed when Pi was present in the bathing media ($[\text{Pi}]$, 1.2 mM, MS flux/SM flux = 1.04, $N = 5$). Identical flux ratios were also observed in the absence of Pi at a $[\text{Ca}^{2+}]$ of 1.25 mM (MS flux = $13.0 \pm 2.0$ SEM mmol/cm$^2 \times 10$ min, $N = 6$; SM flux = $12.4 \pm 1.6$, $N = 6$; flux ratio = 1.03). Therefore, there was no net transport of calcium in adult rats either with or without Pi. Nevertheless, since the PD was unchanged ($0.0 \pm 0.4$ SEM mmv, $N = 9$), flux ratios in adult rats still exceeded the predicted passive ratio of 0.69 so that active transport was present but greatly diminished.

Saturation kinetics. Saturable processes for intestinal calcium absorption have been suggested for the rabbit (13), chick (21), and dog (2) but have not been adequately demonstrated. In the rat, the observed linear concentration-flux relationship has been interpreted by some investigators to indicate that calcium transport in the small bowel does not occur via a carrier-type transport process (4). Saturation kinetics have not been seen over an experimental range of calcium concentrations from $10^{-4}$ to $10^{-1}$ M (4, 22). If these concentrations were one-tenth or less of the $K_m$ for the saturable transport process then the unidirectional Michaelis-Menton equation, $V = V_{\text{max}} \times [S]/K_m + [S]$, would become: $V = V_{\text{max}} \times [S]/K_m + 0.1 K_m$ and can be reduced to the approximation $V = V_{\text{max}} \times [S]/K_m$. The kinetic plots would then be practically inseparable from those predicted by Fick's law of diffusion. In an attempt to saturate the system, unidirectional calcium fluxes were measured over a 100-fold concentration range (0.125-12.5 mM) under the apparently optimal conditions of LCD and Pi-free media. Mucosal-to-serosal flux at calcium concentrations in excess of 3 mM approached a plateau (Fig. 2). Serosal-to-mucosal flux changed proportionally with the calcium concentration, behaving in a manner consistent with simple diffusion or a facilitated diffusion process with a high $K_m$ (Fig. 2). The PD was unchanged over the range of calcium concentrations studied.

Since MS flux was saturable, carrier-mediated transport is indicated. There is undoubtedly a simple diffusional component to both MS flux and SM flux and the magnitude of this process relative to carrier-mediated diffusion and/or active transport cannot be accurately estimated from the present data. A reasonable approximation of the maximal contribution of diffusion to MS flux can be obtained from the passive distribution predicted by the Ussing equation (MS flux = 0.68 $\times$ SM flux). In calculating a $K_m$ and $V_{\text{max}}$ by means of a Lineweaver-Burk plot (Fig. 3) and in plotting the “corrected MS flux” curve of Fig. 2, MS flux was corrected for diffusion by subtracting $0.68 \times$ SM flux at each concentration. The $K_m$ = 1.25 mM and $V_{\text{max}}$ = 59 mmol/cm$^2 \times 10$ min, are not appreciably different from $K_m$ = 1.8 mM and $V_{\text{max}}$ = 80 mmol/cm$^2 \times 10$ min calculated from fluxes not corrected for diffusion. Hopefully, these pairs of values represent the range within which the “true” $K_m$ and $V_{\text{max}}$ for LCD animals exists. Although the calculated $K_m$ and $V_{\text{max}}$ may be descriptive of a single carrier process, because of the several membranes across

### Table 1. Steady-state calcium fluxes across rat duodenum

<table>
<thead>
<tr>
<th>Condition</th>
<th>N</th>
<th>MS Flux, mmol/cm$^2 \times 10$ min</th>
<th>N</th>
<th>SM Flux, mmol/cm$^2 \times 10$ min</th>
<th>Net Flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal + Pi</td>
<td>5</td>
<td>17.1 ± 0.6</td>
<td>4</td>
<td>7.9 ± 2.0</td>
<td>9</td>
</tr>
<tr>
<td>Normal - Pi</td>
<td>6</td>
<td>22.5 ± 2.0</td>
<td>4</td>
<td>7.1 ± 2.0</td>
<td>23</td>
</tr>
<tr>
<td>LCD + Pi</td>
<td>4</td>
<td>29.9 ± 0.1</td>
<td>3</td>
<td>8.2 ± 0.7</td>
<td>26</td>
</tr>
<tr>
<td>LCD - Pi</td>
<td>6</td>
<td>33.7 ± 1.2</td>
<td>3</td>
<td>8.2 ± 0.7</td>
<td>26</td>
</tr>
</tbody>
</table>

Values are means ± SEM. LCD = low calcium diet; Pi = inorganic phosphate. Normal + Pi, MS flux > Normal + Pi, SM flux, $P < 0.005$. LCD + Pi, MS flux > Normal + Pi, MS flux, $P < 0.005$. LCD - Pi, MS flux > LCD + Pi, MS flux, $P < 0.005$. Bath concentrations: $[\text{Ca}^{2+}]$, 1.25 mM; Pi when present, 2.4 mM.
which the measured fluxes occur, the mechanism or mechanisms which these kinetic constants reflect and their location cannot be determined from these experiments alone.

When saturation experiments were performed with normal animals in Pi-free media, MS flux again approached a plateau. Unfortunately, base lines in these experiments were sufficiently different, probably because of seasonal and slight age differences, so that these data cannot be directly compared to LCD saturation kinetics.

DISCUSSION

The present data clearly demonstrate an active calcium transport system in rat duodenum which is not phosphate dependent. Most theories of active transport (other than by pinocytotic processes) presuppose a carrier system as evidenced by saturation kinetics. A saturable process for calcium transport by rat intestine had not previously been demonstrated (4, 22) and this presented difficulty in interpreting results which apparently satisfied thermodynamic criteria for active transport (22). Since Helbock et al. were also unable to demonstrate saturation of transport, and because they did not find any net calcium movement in the absence of Pₐ, they concluded that there was no active transport process for calcium per se in the intestine. In our experiments we were able to satisfy thermodynamic criteria for active transport as well as demonstrate that the transport system was saturable. In addition to saturation of the MS flux process, its unidirectional induction by LCD and enhancement by vitamin D are consistent with an adaptive carrier-mediated transport process.

Part of the conflict between our results and those of Helbock et al. may well be explained by differences in age of the animals used (they used adult rats of 400 g (4), whereas growing animals of 150–200 g were generally used in this study). When we used adult animals we were able to confirm their finding, that there was net duodenal calcium transport in older animals, and the results of others also confirm this finding (11, 12). However, since the transmural Pₐ was not short circuited in our study, according to the Using flux-ratio test active transport of calcium was still present.

In addition, unlike Helbock et al., we were unable to observe a Pₐ dependence for calcium transport either in young or older animals. Even though active calcium MS flux was not dependent on the presence of Pₐ in the present study, there were necessarily other anions present, and the movement of calcium as a counter ion with one of these anions (Cl⁻, HCO₃⁻, or SO₄⁻), although unlikely, cannot be completely eliminated. Also, it is impossible to say whether calcium transport is, at least in part, due to primary active transport, or if it is secondary to the movement of another molecule such as sodium. Because lowering calcium content in the diet (while controlling other dietary minerals) enhanced the active transport of calcium, a specific active system for calcium is indicated. The possible effects of solvent drag on the observed flux ratios were not investigated, but it seems unlikely that solvent drag would be affected by dietary calcium or vitamin D levels and account for the observed variations in calcium transport.

There is a great deal of interest in the possible transport role of the recently isolated vitamin D-dependent calcium-binding protein (CaBP) purified by Wasserman et al. (20, 23). This CaBP was primarily found in the soluble fraction of cell homogenates and a cytoplasmic localization inferred (16, 20). Quite recently, Wasserman reported that preliminary studies attempting to localize the CaBP with fluorescent antibody techniques demonstrated the protein on the brush-border or perhaps the "fuzzy-coat" of intestinal epithelium (R. H. Wasserman; presented in a lecture at the New York Heart Assoc. Symposium on Membrane Transport, New York City, 1968).

The possible membrane or glycocalyx location of the CaBP makes its role as a transport protein (similar to those recently isolated from bacterial cell membranes by osmotic shock (1, 9)) seem more likely than previously indicated by a cytoplasmic localization. The several binding proteins (sulfate, leucine, galactose) isolated from bacteria have binding affinities which reasonably approximate the transport Kₘ for these substrates (10). The binding affinity of the CaBP for calcium is approximately 2.6(10⁻⁴) M⁻¹ (20), while the Kₘ observed in the present study was considerably larger (1.25 mm). Because of the complex nature of the fluxes we studied, it seems quite possible that the observed Kₘ describes the resultant of several processes and that a value much closer to the binding affinity value might be found for the flux across the mucosal cell membrane alone. However, the possibility that CaBP performs other than a primary transport function cannot be eliminated. It is worthy of note, however, that the observed Kₘ for calcium transport is within one order of magnitude of the reported Kₘ values for other mammalian cation transport systems (15).

Calcium absorption in vivo is responsive to the animal's needs for calcium primarily for the formation of bone (8). Schachter et al. demonstrated the greatest ⁴⁴Ca ratios in everted gut sacs from younger animals, pregnant animals and animals on LCD (11). While calcium absorption appears to be responsive to the animal's needs for calcium, the mechanism involved in this adaptation has not been clear. Our results show that LCD unidirectionally increases calcium MS flux and this increase represents a regulation of the active transport process. Such an effect could occur either by a direct increase in the active process or through increased availability of substrate to the "pump" if this were the rate-limiting step.

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REFERENCES


