Calmodulin-regulated, ATP-driven Calcium Transport by Basolateral Membranes of Rat Small Intestine*

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Uptake of calcium by a membrane fraction enriched in basolateral plasma membrane vesicles from rat small intestine has been investigated using a rapid filtration technique. Calcium accumulation is stimulated by ATP and released by the calcium ionophore A23187. Kinetic studies indicate a calcium concentration of 28 mM for half-maximal stimulation of ATP-driven uptake. Transport is minimally inhibited by oligomycin and ouabain but is reduced 40% by lanthanum and abolished by 50 μM vanadate. The ATP-dependent calcium uptake is stimulated by the calcium regulatory protein, calmodulin, in a dose-dependent fashion. Calmodulin increases both the maximal transport rate and the calcium affinity of the transport mechanism. These results are consistent with the existence of an ATP-dependent, calmodulin-regulated calcium transport mechanism in the basolateral plasma membrane of intestinal epithelial cells, and suggests that calmodulin may modulate transepithelial calcium absorption in addition to controlling cytosolic calcium levels.

Calcium transport mechanisms in red blood cells, mitochondria, and sarcoplasmic reticulum have all been described in considerably greater detail than the mechanism driving calcium transport in epithelial systems. Active calcium transport by the small intestine has been well documented for absorptive fluxes (18), and recently both voltage-dependent and sodium-dependent modes of mediated transport have been described for rat ileum (16, 17). The specific mechanisms for intestinal calcium transport derived from intact tissue studies have, however, remained relatively obscure. The possibility of elucidating the mechanism and regulation of transepithelial calcium flux in isolated membrane preparations holds greater promise than intact tissue methodologies, since both passive driving forces as well as metabolic energy supplies can be more precisely controlled thereby. Such studies have yielded considerably greater detail of the calcium transport events at the luminal or brush-border membrane in chick small intestine (26) as well as in the basolateral membrane of kidney tubular epithelium (7). The current investigation presents results suggesting the participation of an ATP-dependent calcium pump at the basolateral membrane of rat small intestinal epithelium and implicates the calcium regulatory protein, calmodulin, in the control of cytosolic calcium through calcium efflux from the intact enterocyte.

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EXPERIMENTAL PROCEDURES

Basolateral Membrane Preparation—Sprague-Dawley male rats, 125-250 g, raised on standard Purina rat chow, were sacrificed by cervical dislocation, and the proximal 60 cm of each small intestine was removed. Isolated epithelial cells from 3 rats were prepared by a modification of the technique of Murer et al. (15). After flushing the intestine with 150 mM NaCl, a buffered, isotonic 25 mM citrate solution was employed to free epithelial cells. Following a 15-min incubation in the citrate buffer at 37°C, a solution of 5 mM EDTA, pH 7.4, was used to harvest the epithelial cells, combined with gentle massage of the intestinal segment. Enterocytes (10-15 g) were collected at 500 x g for 10 min, followed by homogenization in a standard Dounce homogenizer in 75 ml of a sucrose buffer containing 250 mM sucrose, 10 mM triethanolamine hydrochloride, pH 7.5, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 mM EDTA. A spontaneously formed Percoll density gradient, as described by Scalera et al. (28), was used to isolate the basolateral membranes. Low speed centrifugations were carried out in a Sorvall RC 2 centrifuge with a Sorvall SS-34 rotor, while the Percoll gradient and Percoll recovery steps were performed in a Beckman L-265B ultracentrifuge using a fixed-angle Beckman 30 rotor.

The above homogenate was centrifuged at 2,500 x g for 15 min, and the supernatant was recovered for centrifugation at 20,500 x g for 20 min. The fluffy layer of the resulting pellet was then resuspended in 22.5 ml of the above sucrose buffer and homogenized with 20 strokes of a Potter-Elvehjem homogenizer at 1,400 rpm. Following homogenization, 2.5 ml of commercial Percoll (Sigma) was added with 2 additional strokes of the Potter-Elvehjem. This preparation was then spun at 48,000 x g for 25 min, and the gradient was fractionated with 60% sucrose as described by Scalera et al. (28). The fraction typically yielding the highest enrichment in (Na-K)-ATPase1 appeared in a 4-ml volume at a specific density approximately 1.07 gm/ml. Percoll was separated from the recovered fractions by centrifugation in a swinging bucket rotor at 48,000 x g for 30 min after a 30-fold dilution with the above sucrose buffer. The membranes formed a dense layer over the residual Percoll pellet under these conditions and were typically resuspended in a buffer containing 250 mM sucrose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/Tris, pH 7.5.

Assay of Marker Enzymes—(Na-K)-ATPase activity was measured by a modification of the method of Siegel and Goodwin (30) with the determination of inorganic phosphate by the method of Chen et al. (2). Activity is defined as the difference between total adenosine triphosphatase and ouabainized or K-free activities. Succinic dehydrogenase and NADPH cytochrome c reductase were assayed spectrophotometrically by the methods of King (10) and Mircheff and Wright (14), respectively. Diisacharidase activity was determined using the method of Dahlquist (5), and protein levels were measured using the technique of Lowry (12) with bovine serum albumin as the standard.

Calcium Transport Technique—Purified membranes were preincubated for 30 min at 25°C in a solution of the following composition: 120 mM mM KCl, 5 mM MgCl2, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/Tris, pH 7.5. Uptake of calcium at 25°C was initiated with the addition of 4Ca and 48Ca in the above buffer, with a final activity of 8-10 x 107 cpm/ml and a protein concentration of 1

1 The abbreviations used are: (Na-K)-ATPase, sodium-potassium-stimulated ATPase; ER, endoplasmic reticulum; X, apparent half-maximal saturation constant.
approximately 0.5 mg/ml. Free calcium concentrations below 10 μM were maintained with EGTA buffering as detailed by Pershadsingh and McDonald (24) for pH 7.5 (Kw = 6.76 × 10^-10 M). Typically, total calcium was held at 1 mM and EGTA was increased to yield the desired free calcium concentrations. ATP was added as the Tris salt (5 mM) with 45Ca. At appropriate times, 100-μl samples were removed in duplicate and immediately diluted in 5 ml of ice-cold stopping medium. The composition of the stopping medium was identical to the incubation medium with the exception that all Ca was omitted, and 1 mM EGTA together with [3H]β-mannitol (3 × 10^6 cpm/ml) and 2 mM D-mannitol was added. This mixture was immediately filtered through a 0.45-μm Millipore filter (HAWP2500) which had been previously washed with 5 ml of incubation medium. The filter was then washed with two 3-ml aliquots of ice-cold incubation medium. Isotopes retained by the filter were counted in a Beckman Model 350 liquid scintillation spectrometer using a toluene-Triton scintillation mixture, Formula 950A (New England Nuclear). Tritium counts provided an index of retained incubation medium. The stopping and washing procedure was routinely completed in 10 s for each sample. Nonspecific binding of Ca to the Millipore filter was routinely assessed in the absence of membrane protein and seldom exceeded 5% of the experimental values.

Calmodulin, isolated from Electrophorus electricus electrophores using the technique of Childers and Siegel (4) was kindly provided by Dr. G. Jagiello, Columbia University. The purity of the calmodulin preparation was established by sodium dodecyl sulfate-gel electrophoresis where the protein was resolved as a single band. The stimulation of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase by Graf and Penniston (23) was used as the criterion for identity of the protein. The calmodulin ionophore A23187 was provided by Dr. R. L. Hamill of Eli Lilly, Indianapolis, IN. Isotopic species were obtained from New England Nuclear. Tris-ATP (vanadate-free) was supplied by Sigma. Results are expressed as mean ± S.E. for the number of separate preparations indicated.

RESULTS

The membranes harvested from the Percoll gradient yielded fractions with distinct distribution of brush-border sucrase and basolateral (Na-K)-ATPase markers as illustrated in Table I. In all preparations used for transport experiments, (Na-K)-ATPase was assayed to determine purification with respect to homogenate. Values for succinic dehydrogenase and NADPH cytochrome c reductase and markers for mitochondrial and microsomal membranes, respectively, are also presented in Table I. Note that both succinic dehydrogenase and NADPH cytochrome c reductase are significantly reduced by 4- and 5.1-fold, respectively, during purification of the membrane fraction. Contaminants, presumably endoplasmic reticulum, are slightly enriched (2.6-fold) but remain significantly less than the 6.5-fold elevation in (Na-K)-ATPase activity, which is confined to the basolateral plasma membrane. While the increase in (Na-K)-ATPase activity relative to homogenate is somewhat greater than those observed in the absence of separation by free-flow electrophoresis. Thus, the final membrane preparation is specifically enriched in (Na-K)-ATPase, which suggests that the preferential fraction is basolateral plasma membrane, although small contributions from another membrane fraction, presumably endoplasmic reticulum, cannot be completely excluded.

The stimulation of calcium uptake by Tris-ATP is illustrated in Fig. 1. The presence of ATP results in more than a 6-fold stimulation of initial calcium uptake and accumulation at steady state. The inclusion of the calcium ionophore A23187 in the presence of ATP results in a rapid entry of calcium into the membrane vesicles but precludes calcium accumulation to levels appreciably greater than those observed in the absence of ATP. It is assumed that a large fraction of the vesicle population is oriented inside out, thereby allowing ATP access to the membrane-bound hydrolytic site. These results suggest the generation of an ATP-dependent calcium gradient which is readily dissipated by an increase in vesicle calcium conductance. In the absence of ATP, vesicle-associated calcium is sequestered to some extent by membrane-binding sites. The apparent intravesicular volume of these vesicles as assessed by steady state L-glucose accumulation is 0.9 ± 0.11 ml/mg protein (n = 3). This volume would accommodate 0.09 nmol/mg protein of free Ca at 100 μM bathing calcium. Since the observed value for vesicle-associated calcium in the absence of ATP (Fig. 1) is in excess of 1 nmol/mg protein, significant binding of calcium to the vesicle membranes must occur. In the presence of ATP, further binding of calcium to similar sites cannot be excluded from the present interpretation. Although the effect of A23187 suggests that the ATP-dependent increase in calcium accumulation is associated with either an unbound pool or with intravesicular binding sites, which have significantly lower calcium binding affinity than those observed in the absence of ATP.

Further evidence for the intravesicular deposit of the ATP-dependent calcium component is presented in Fig. 2, where calcium efflux from vesicles preincubated with 45Ca is illustrated. In the absence of ATP, vesicle-associated calcium is unperturbed by the addition of calcium ionophore A23187, consistent with a passive equilibration of calcium across the vesicle membranes. On the other hand, the ionophore induces a rapid initial release of 30% of the ATP-dependent calcium component, followed by a slower progressive release approach.

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### Table I

<table>
<thead>
<tr>
<th>Enzyme marker activity of isolated membrane fractions</th>
<th>Homogenate</th>
<th>Final membrane fraction</th>
<th>ΔA/mg.min</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Na-K)-ATPase</td>
<td>63.2 ± 5.0</td>
<td>410.0 ± 27.6</td>
<td>0.0056 ± 0.0020</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>7.2 ± 12.4</td>
<td>17.4 ± 9.4</td>
<td>0.0148 ± 0.0049</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>77.5 ± 20.1</td>
<td>15.2 ± 4.5</td>
<td></td>
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</tbody>
</table>

*Thirty separate preparations were assayed for (Na-K)-ATPase and 5 preparations were assayed for the remaining markers.*
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**Fig. 2.** Effect of calcium ionophore A23187 on vesicle-associated Ca²⁺. In the presence and absence of 5 mM ATP. Vesicles were loaded as in "Experimental Procedures" in 100 μM total Ca²⁺ (no EGTA) for 60 min in the presence or absence of 5 mM ATP. At time 0, 10 μM A23187 was added to the vesicles. Vesicle Ca⁴⁺ is normalized to time 0 values in the presence of ATP, and the values represent the means ± S.E. of three experiments.

**Fig. 3.** Kinetics of ATP-dependent Ca²⁺ uptake. Uptake of Ca²⁺ measured as in "Experimental Procedures" at pH 7.5 with total [Ca] = 1 mM and [EGTA] = 1.01–2.5 mM. J⁹Ca represents the difference between calcium uptake in the presence and absence of ATP. The results are the mean ± S.E. of four experiments.

**Fig. 4.** Response of ATP-dependent Ca²⁺ uptake (J⁹Ca) to varying doses of calmodulin. Calcium uptake (3 min) was measured as in "Experimental Procedures" in the presence of 1.01 mM EGTA and 1.00 mM total Ca (EGTA) = 1.01–2.5 mM. Calmodulin was present at 5 μg/ml during both 30-min preincubation and incubation. No effect of calmodulin on Ca²⁺ uptake in the absence of ATP could be detected (data not shown). Results are plotted as the fractional stimulation of Ca²⁺ uptake in response to calmodulin for three experiments where mean ± S.E. are indicated. J⁹Ca, difference between calcium uptake in the presence and absence of ATP.

**Fig. 5.** Kinetics of ATP-dependent calcium uptake in response to calmodulin. Uptake of calcium measured as in Fig. 3 where calmodulin (0 μg/ml) was present or absent during both 30-min preincubation and uptake. Results are presented as the mean ± S.E. of three experiments. J⁹Ca, difference between calcium uptake in the presence and absence of ATP.

The transport mechanism suggests that it may function efficiently at proposed cytosolic calcium concentrations of 1 μM or less.

Several inhibitors have been employed to further characterize ATP-dependent calcium uptake. In the presence of 120 mM KCl, 10 mM 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid/Tris, and 5 mM MgCl₂ inside and out, ATP-dependent calcium uptake at 3 min ([Ca] = 100 μM) was not inhibited by 1 mM ouabain and was decreased 12% by oligomycin. On the other hand, 0.1 mM lanthanum decreased uptake by 40%, and removal of Mg²⁺ completely abolished ATP-driven uptake.

The vanadate effect together with the oligomycin result suggests that the transport system is probably of plasma membrane origin rather than from a mitochondrial contaminant, since plasma membrane (Ca²⁺-Mg²⁺)ATPases appear to be vanadate-sensitive while those of mitochondrial origin are not.

**DISCUSSION**

Active accumulation of calcium has been demonstrated in a rat small intestinal membrane fraction specifically enriched in basolateral plasma membranes. The saturable transport process requires both magnesium and ATP for the generation of calcium gradients dissipated by the calcium ionophore A23187, and is completely inhibited by vanadate. This evidence suggests that calcium traverses the vesicle membrane...
via a specific transport mechanism in response to ATP. The uptake process is characterized by a calcium $K_t$ of 28 ± 3 nm with a maximal transport rate of 2.55 ± 0.26 nmol/mg · 3 min. The affinity is reasonably close to the value observed for the (Ca$^{2+}$+Mg$^{2+}$)-ATPase of this preparation, 71 ± 6 nmol/mg · 3 min, measured under similar conditions (data not shown), and suggests the identity of the ATPase and the calcium pump. The relatively high affinity of this preparation for calcium is only slightly higher than values reported for adipocyte (22, 24) and pancreatic islet (23) plasma membrane also investigated at pH 7.5. On the other hand, the $K_t$ of the calcium pump of red-blood cell plasma membranes has been reported as 4 μM at pH 7.0 (29), and a $K_t$ of 0.5 μM has been reported for intestinal calcium ATPase (6). The difference between these $K_t$ values may be real, although it is possible that the critical pH dependence of the EGTACa association constant is a contributing factor leading to widely divergent calculated free-calcium concentrations. The more critical issue is that the transport affinity of the intestinal system is below estimates of cytosolic free calcium, which range from 50 to 1000 nm, thus providing the pump with the requisite affinity at physiological calcium activities.

A significant stimulation of calcium uptake by calmodulin has been demonstrated for this basolateral membrane-enriched preparation and represents the first such observation in an epithelial system. The 35% increase in maximal transport rate together with the half-maximal calmodulin dose of 0.5 μM suggests several possibilities when compared to the activation observed in red blood cells (20). In intact red cells, stimulation of calcium transport is often greater than 2-fold at a concentration of 1 μM/ml calmodulin (8, 25). The difference in response of the intestinal preparation to calmodulin may be the result of a variety of factors including endogenous unreleased calmodulin, as reported for microsomes of smooth muscle (34), an inherently different regulatory mechanism, or a failure to establish the optimal conditions for the calmodulin response. Clear discrimination among these possibilities may be possible at the present time. However, inhibition of ATP-dependent calcium uptake in the absence of calmodulin by 100 μM chlorpromazine (data not shown) in our preparation suggests a role of tightly bound calmodulin despite use of EDTA washes. In addition, use of 120 mM KCl in the control buffer may decrease the stimulatory efficiency of calmodulin as in cardiac sarcoplasmic reticulum (11). Thus, the mechanism of calmodulin interaction with the epithelial calcium pump will require further examination before comparisons are made with other plasma membrane systems such as the red cell or cardiac sarcolemma (32).

Inhibition of the calcium pump by vanadate and lanthanum suggests that the site of action is at the cytosolic membrane face. Lanthanum at 100 μM inhibits more than 80% of red cell calcium transport (27) and 100% of sarcoplasmic reticulum ATP utilization (3), but decreases intestinal basolateral calcium transport by only 40%. The intestinal transport mechanism may be inherently different than that of the red cell, and sarcoplasmic reticulum and/or lanthanum may not gain access to all inhibitory sites since the primary site in the red cell appears to be at the external membrane face (27). The abolition of calcium transport by vanadate in the basolateral preparation is consistent with the observation of O'Neal et al. (21) and Nelson and Blaustein (19) for a variety of plasma membrane (Ca$^{2+}$+Mg$^{2+}$)-ATPases, and provides further evidence for the absence of significant mitochondrial contamination since vanadate has been reported (21) to have a negligible effect on mitochondrial (Ca$^{2+}$+Mg$^{2+}$)-ATPase.

Significant contamination of the basolateral membrane-enriched mitochondrial fraction by membranes containing NADPH cytochrome c reductase, presumably ER, suggests that some fraction of the calcium transport activity may be associated with this contaminant. Calcium transport dependent on ATP has been reported for the endoplasmic reticulum of adipocytes (1), although calcium uptake by this preparation could not be stimulated by calmodulin. If intestinal ER is refractory as well, then the stimulation of calcium uptake induced by calmodulin in the present study may be limited by an unresponsive ER subtraction of the total calcium-transporting population. Resolution of this question requires isolation of a relatively pure ER vesicle fraction whose behavior may be contrasted with that of the basolateral-enriched preparation. Characterization of an ATP-dependent sequestration of calcium by ER is of comparable interest to that of basolateral plasma membrane, and the present observations provide the impetus for investigation of both systems in purer isolated fractions.

Significant stimulation of ATP-dependent calcium transport by calmodulin, however, does suggest that this regulatory protein could be involved in the control of both cytosolic free calcium concentration and the rate of transepithelial calcium transport. Cytosolic calcium, its role in intestinal secretory states, and its control by calmodulin have all been implicated recently (8, 31) in the control of electrolyte transport events in the small intestine. Increases in cytosolic calcium may be required for calmodulin activation of electrolyte secretion associated with some diarrheal states (9). The potential decrease in cytosolic calcium induced by calmodulin-stimulated transport in basolateral membranes suggests that calmodulin may function not only as an activator of cell function in association with calcium, but also that under appropriate conditions it may be self-limiting by inducing a decline in cytosolic calcium. In addition, the role of calmodulin in the regulation of transepithelial calcium absorption must be considered; undoubtedly a calmodulin extrusion mechanism at the basolateral aspect of the intestinal enterocyte must be responsible for some component of transepithelial calcium transport. The elucidation of the fine control of such a mechanism together with possible interactions with vitamin D metabolites should now be accessible with isolated membrane techniques such as the one described here.

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REFERENCES
Calmodulin-regulated Epithelial Calcium Transport