

Identification of an Intestinal Sodium and Calcium-Dependent Phosphatase Stimulated by Parathyroid Hormone

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ABSTRACT Previous reports suggest that the site of the energy-dependent intestinal calcium transport against an electropotential and concentration gradient is located along the basal-lateral membrane of the mucosal cell. Accordingly, basal-lateral membranes were prepared from rat intestinal homogenates in order to identify the enzyme mediating this step in the transport process. An alkaline phosphatase was delineated which utilized ATP as a substrate and was dependent on both Na^+ and Ca^{++} with optimum enzyme activity at 200 mM and 0.04 mM, respectively. Furthermore, the activity of the enzyme was demonstrated to decrease with the advance in age of the animal and to decrease with removal of the parathyroid glands, consistent with a decreased rate of ^{45}Ca release from mucosal cells under the same experimental conditions. Calcium binding to basal-lateral membrane fragments was also sodium dependent and enhanced by the prior administration of parathyroid extract. The consistent correlation between the rate of calcium transport across the basal-lateral membrane of the mucosal cell and the activity of this Na, Ca-dependent phosphatase under a variety of experimental conditions suggest that this enzyme may mediate the parathyroid hormone-sensitive active transport of calcium across the intestine.

INTRODUCTION

The movement of calcium across the intestinal mucosal epithelium involves a series of events capable of accumulating calcium against an electropotential and concentration gradient. An important, if not the rate-limiting, step in this transport process is the movement of

calcium across the basal-lateral membrane of the epithelial cell (1). This step requires sodium (2) but is independent of net sodium flux and is not inhibited by inhibitors of the Na, K-exchange pump (2-4). Parathyroid hormone, as demonstrated in the preceding report (3), stimulates this sodium-dependent step. From these observations, a number of models can be constructed for the mechanism of calcium transport which are analogous to cation transport across other cell membranes. These cation transport processes are characterized by membrane-associated enzyme complexes which specifically interact with one or more cations to initiate the sequence of events of the transport process. In addition these enzyme complexes hydrolyze ATP which provides the driving force for the translocation of the cation across the cell membrane.

This report concerns the identification of an alkaline phosphatase localized to the basal-lateral membrane of the intestinal mucosal epithelial cells which is activated by sodium and calcium. The activity of this enzyme is specifically enhanced by parathyroid hormone.

METHODS

Preparation of animals. Intestinal mucosal epithelial tissue was obtained from male Wistar rats $3\frac{1}{2}$ wk of age. The animals were maintained on a calcium-deficient diet (General Biochemical Div., Mogul Corp., Chagrin Falls, Ohio) for 3 days and fasted overnight before sacrifice. In evaluating the influence of parathyroid hormone, the animals were parathyroidectomized by hot wire cautery or sham operated 24 h before sacrifice. The parathyroidectomized animals were then maintained on a 1.9% calcium, 0.4% phosphorus diet and the parathyroid-intact animals were continued on the calcium-deficient diet with 0.4% phosphorus. The influence of exogenous parathyroid hormone was studied as previously described (3). Parathyroidectomized animals were given subcutaneously either 30 U of parathyroid extract (Eli Lilly and Co., Indianapolis, Ind.) or 0.3 ml of 0.2% phenol and 1.6% glycerin in saline 4 h before sacrifice. To simulate the rise in serum calcium fol-

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lowing hormone injection, the hormone-deficient control animals were given by intraperitoneal injection 17 and 34 μ mol of calcium gluconate at 3.5 and 1.0 h, respectively, before sacrifice. The hormone-treated animals were given an equivalent dose of sodium gluconate.

Preparation of membranes. The first 14 cm of the proximal intestine was everted and washed and the mucosa scraped from the underlying submucosal tissue with a spatula. Approximately 2.0–2.5 g of mucosal epithelium was suspended in 100 ml of 5 mM EDTA, pH 7.4, and homogenized in a VirTis homogenizer (model 23, VirTis Co., Inc., Gardiner, N. Y.) for 60 s at the lowest setting, then for 20 s at a setting of 20. The homogenate was filtered through number 9 then number 25 bolting silk (Dufour, New York). The filtrate was centrifuged twice at 750 *g* in a Sorvall centrifuge (Model RC2-B, Ivan Sorvall, Inc., Newtown, Conn.) and the resulting pellet was washed three times with 2.5 mM EDTA, pH 7.4. The final pellet constituted the brush border fraction. Purified brush border fragments were obtained from this fraction by the method of Forstner, Sabesin, and Isselbacher (5). The basal-lateral membrane fragments were sedimented from the supernate of the initial 750 *g* centrifugation by a second centrifugation at 10,000 *g* for 10 min. This pellet, containing mitochondria and cell membrane fragments, was suspended in 1 mM EDTA, pH 7.4, and allowed to stand overnight. The suspension was then layered over a discontinuous sucrose gradient of 25 and 30% as described by Quigley and Gotterer (6). The gradient was centrifuged at 64,000 *g* for 90 min. The basal-lateral membrane fragments sediment to the 25–30% sucrose interphase. This fraction is withdrawn and suspended in 30 mM imidazole-histidine buffer adjusted to pH 7.4 with 30 mM Tris base (IHT buffer).¹ The pellet containing the mitochondria was suspended in the same buffer and stored at 4°C. The suspension of basal-lateral membrane fragments was sedimented at 100,000 *g* for 45 min, then resuspended in 30 mM IHT buffer, pH 7.4, containing 0.04 mM CaCl_2 and stored at 4°C. In studying the influence of calcium on the enzyme, the membrane preparation was resuspended in the same buffer containing 0.004 mM CaCl_2 . The membrane suspension could not be frozen without complete loss of activity. At 4°C, approximately 25% of the initial activity was lost every 24 h. In the absence of calcium this loss of activity was greatly accelerated and could only be partially restored by the subsequent addition of calcium before assay.

Enzyme assays. Phosphatase activity was measured in the IHT buffer, pH 7.4, at a final concentration of 50 mM. Tris-ATP (Sigma Chemical Co., St. Louis, Mo.) was added at a final concentration of 2 mM. The appropriate cation or cations and inhibitors were added to the assay in a fixed volume of distilled water to give a volume of 80 μ l. The reaction was initiated with the addition of 20 μ l of the enzyme preparation to give a final volume of 100 μ l and incubated for 20–40 min at 37°C. Aliquots were removed at 10-min intervals for analysis of inorganic phosphate released from the hydrolysis of ATP by a modification of the method of Fiske and SubbaRow (7). The appropriate blanks were included to correct for nonenzymatic hydrolysis of organic phosphate. The concentration of the enzyme was adjusted to give linear kinetics for the duration of incubation. Na, Ca-dependent phosphatase activ-

ity as referred to in this report is the difference between the activity of the enzyme preparation in the presence of 160 mM sodium, 0.04 mM calcium and the activity of the enzyme preparation in the presence of 0.04 mM calcium in the absence of sodium. The (Na, K) Mg-ATPase activity was determined in the presence of 2 mM magnesium, 120 mM sodium, and 15 mM potassium and is expressed as the difference between the activity in the presence and absence of the monovalent cations. The ouabain-sensitive component was assayed in the presence of 0.5 mM ouabain (Sigma Chemical Co.) and expressed as the difference between the (Na,K) Mg-ATPase activity in the absence and presence of the inhibitor. Ethacrynic acid was kindly supplied by Merck, Sharp & Dohme, West Point, Pa.). All phosphatase data were expressed as nanomoles inorganic phosphate released per milligram membrane protein (8). Sucrose activity was determined with the conditions described by Dahlqvist (9) and cytochrome oxidase by the method of Smith (10).

Calcium binding assay. Brush border and basal-lateral membrane suspensions with protein concentrations of 1.0–2.0 mg/ml were equilibrated with ^{45}Ca , 0.001 $\mu\text{Ci/ml}$ in 30 mM IHT buffer, pH 7.4, containing 0.1 mM magnesium, 0.004 mM calcium, and 160 mM sodium when indicated. Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) was prepared as described by Wasserman and Taylor (11) and equilibrated with 0.1 mM magnesium, pH 7.4. The Chelex-100 resin was suspended in the 0.1 mM magnesium buffer at a ratio of 1 part resin to 4 parts buffer. 400 μ l of the Chelex-100 suspension was delivered to a test tube containing 200 μ l of the membrane suspension with the appropriate cations and ^{45}Ca . The reaction mixture was stirred on a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.) at regular intervals for 10 min. The resin was then sedimented at 750 *g* for 5 min and an aliquot of the supernate was taken for protein and radioactivity determinations.

RESULTS

The various membrane fractions obtained by a modification of the method of Quigley and Gotterer (6) were characterized with respect to sucrose, cytochrome oxidase, and ouabain-sensitive (Na, K) Mg-ATPase activity as enzyme markers for the brush border, mitochondria, and basal-lateral membranes, respectively (Fig. 1). That fraction designated as the basal-lateral membrane fraction demonstrated significant enhancement of ouabain-sensitive (Na, K) Mg-ATPase activity with respect to the principle contaminating elements, the brush border, and mitochondria. This segregation of the ouabain-sensitive (Na, K) Mg-ATPase activity with the basal-lateral membranes is consistent with the observations of others (12) and, therefore, provides a convenient marker for this membrane fraction. In only the basal-lateral membrane fraction could stimulation by sodium of calcium-dependent phosphatase activity be demonstrated at pH of 7.4. The sodium and calcium-dependent phosphatase activity (Na, Ca-dependent phosphatase) of the enzyme preparation was dependent on the concentration of the preparation and was partially inactivated by preincubating the enzyme prepa-

¹ Abbreviation used in this paper: IHT buffer, 30 mM imidazole-histidine buffer adjusted to pH 7.4 with 30 mM Tris base.

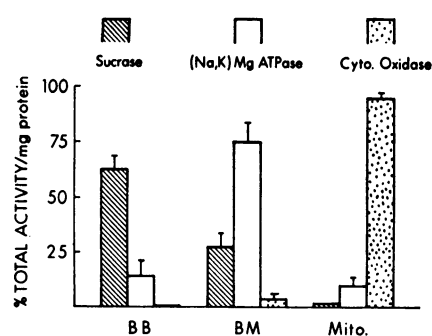


FIGURE 1 The preparation of basal-lateral membranes as described in the text yields three particulate fractions identified as the brush border fraction (BB), the basal-lateral membrane fraction (BM), and the mitochondrial fraction (Mito.). Each fraction was characterized with respect to sucrose activity, ouabain-sensitive (Na, K) Mg-ATPase activity ((Na, K) Mg ATPase), and cytochrome oxidase activity (Cyto. Oxidase). The enzyme composition of each fraction was expressed as a percent of the total enzyme activity per milligram protein present in the three fractions. The vertical bars indicate the SEM of four membrane preparations.

ration in trypsin (0.1 mg/ml) for 30 min at 37°C (Fig. 2).

The cation dependence of the enzyme activity was investigated in some detail. First, at pH of 7.4 and a calcium concentration of 0.04 mM, maximal stimulation by sodium of calcium-dependent phosphatase activity was observed at a sodium concentration of 200 mM. The requirement for magnesium of the Na, Ca-dependent phosphatase was examined at concentrations of 160 mM sodium and 0.04 mM calcium. Although 0.1

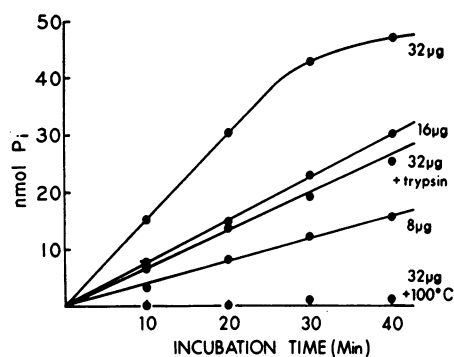


FIGURE 2 Varying dilutions of a basal-lateral membrane preparation, expressed as micrograms of protein, were delivered to reaction tubes containing the appropriate cations in 40 mM imidazole-histidine-tris buffer, pH 7.4, and tris-ATP at a final concentration of 2 mM. The undiluted membrane preparation was preincubated with either trypsin (0.1 mg/ml) or heat-inactivated trypsin. A second aliquot of the undiluted membrane preparation was heated at 100°C for 15 min. The inorganic phosphates (P_i) released by the enzyme activity from the hydrolysis of ATP is expressed as a function of time after initiation of the reaction.

TABLE I
Effect of Mg^{++} on the Na, Ca-Dependent Phosphatase Activity

| Cation addition | | Total Phosphatase Activity | |
|---|--------|----------------------------|-------------|
| Ca^{++} | Na^+ | + Mg^{++} | - Mg^{++} |
| <i>nmol P_i min/mg protein</i> | | | |
| + | - | 67±1.0 | 52±1.2 |
| + | + | 91±1.3 | 80±2.1 |
| - | + | 65±1.9 | 57±1.1 |
| - | - | 50±2.4 | 43±2.2 |

The assays were performed in the IHT buffer, pH 7.4, containing 2 mM ATP. To this buffer was added at a final concentration Ca^{++} , 0.04 mM; Na^+ , 160 mM; Mg^{++} , 0.1 mM as indicated in the table. The phosphatase activity represents the mean±SEM of four determinations from two enzyme preparations.

mM magnesium stimulated basal phosphatase to a greater extent than 0.04 mM calcium, the Na, Ca-dependent phosphatase activity (the difference between that enzyme activity obtained in the presence of sodium and calcium and that activity obtained in the presence of calcium alone) in the absence of magnesium was 28 compared to 24 nmol P_i /min/mg protein in the presence of magnesium (Table I). Thus the addition of magnesium is not required for that activity of the enzyme preparation which is dependent on both sodium and calcium. In order to ascertain the optimum calcium concentration for the Na, Ca-dependent phosphatase, the enzyme preparation was assayed in the presence of 0.1 mM magnesium and 160 mM sodium (Table II). Un-

TABLE II
The Calcium Dependency of the Phosphatase

| Concn cation added | Na-dependent activity | | Specific Na, Ca-activity |
|--------------------|---|-------------|--------------------------|
| | + Ca^{++} | + Mg^{++} | |
| <i>mM</i> | <i>nmol P_i/min/mg protein</i> | | |
| 0.001 | 26.8±3.9 | 24.8±3.4 | 2.0 |
| 0.004 | 43.6±2.3 | 25.6±4.2 | 18.0 |
| 0.04 | 60.0±2.5 | 29.5±2.8 | 30.5 |
| 0.4 | 34.3±3.5 | 27.9±3.2 | 6.4 |
| 4.0 | 28.0±2.8 | 34.0±2.8 | 0 |

The enzyme assays were performed in the IHT buffer, pH 7.4, containing 2 mM ATP, and 0.1 mM Mg^{++} . To this buffer was added either Mg^{++} or Ca^{++} at the final concentration as indicated. The phosphatase activity represents the mean of 4 membrane preparations performed in duplicate. The Na^+ -dependent activity was determined as the increment in activity in the presence of 160 mM Na^+ at each concentration of the divalent cation. The specific Na, Ca-activity is the difference in the Na^+ stimulation in the presence of Ca^{++} from that in the presence of Mg^{++} as the sole divalent cation.

der these conditions, all sodium-stimulated nonspecific divalent cation-dependent phosphatase activity would be activated. Increasing concentrations of calcium or the same molar concentration of magnesium were added to the reaction buffer before assay. That activity which could be attributed to a specific Na, Ca-dependent phosphatase was determined by subtracting the stimulation obtained by the addition of magnesium from that stimulation of enzyme activity resulting from the equimolar addition of calcium. The difference, or the specific Na, Ca-dependent activity, is presented in Table II. Thus significant and specific activation of the enzyme is demonstrable at calcium concentrations of 0.004–0.4 mM. Examination of the pH dependence of Na, Ca-dependent phosphatase revealed an optimum pH for the reaction at 8.3 in the presence of 2 mM ATP. Of the limited number of substrates tested in the Na, Ca-dependent phosphatase reaction at pH 7.4, ATP was preferred substrate (Table III).

The correlation between physiologic alterations of calcium transport and the activity of the Na, Ca-dependent phosphatase of the basal-lateral membrane was investigated. The rate of ^{45}Ca release was significantly decreased in cells obtained from older animals. This decreased release was associated with a decrease in the

TABLE III
Substrate Specificity

| Substrate | Na, Ca-dependent activity |
|-----------|-----------------------------------|
| | <i>nmol Pi/min/mg protein</i> |
| ATP | 65.2±1.8 |
| GTP | 42.4±0.6 |
| PPP | 12.7±1.5 |
| CTP | 4.1±2.0 |

The phosphatase activity was determined as described in the text. The following substrates were determined at a final concentration of 2 mM, pH 7.4; adenosine triphosphate (ATP), guanosine triphosphate (GTP), paranitrophenyl phosphate (PPP), and cytosine triphosphate (CTP). The phosphatase activity represents the mean±SEM of four experiments performed in duplicate.

specific Na, Ca-dependent phosphatase activity. Similarly, parathyroidectomy also decreased the release of ^{45}Ca from the mucosal cell and the administration of parathyroid extract to the parathyroidectomized animal partially restored the rate of ^{45}Ca release. These changes in ^{45}Ca release again paralleled the changes in the ac-

TABLE IV
Influence of Age and Parathyroid Activity on Transport and Enzyme Activity

| | Release rate | Na, Ca-phosphatase | Na, K-ATPase | Ratio |
|----------------------|-----------------|-----------------------|---------------|-------|
| Age | | | | |
| 3.5 wk | 0.53±0.01 (10) | 54.8±9.8 | 113.5±3.7 (2) | 0.48 |
| 6.5 wk | 0.36±0.01 (10)* | 17.5±2.5* | 84.0±1.3 (2) | 0.21 |
| Parathyroid activity | | | | |
| PTHx | 0.31±0.02 (16) | 18.8±4.8 | 91±5.2 (10) | 0.21 |
| Intact | 0.55±0.01 (12)‡ | 37.0±2.3‡ | 87±4.2 (6) | 0.42 |
| PTHx + PTH | 0.48±0.02 (16)§ | 31.7±2.8§ | 79±2.9 (4) | 0.40 |

The correlation between the release of ^{45}Ca from the mucosal cell and enzyme activity of the basal-lateral membrane were compared under the influence of age and parathyroid activity. The rate of release is expressed as that fraction of the initial radioactivity released in 6 min after loading the cells for 6 min with the isotope. The parathyroid studies were the same as those described previously (3). Basal-lateral membranes were obtained from pools of 10 animals each prepared in the same manner as those animals used in the corresponding ^{45}Ca release studies. The phosphatase activity is expressed as nanomoles inorganic phosphate released from ATP per minute per milligram protein. The values are the mean±SEM of four determinations of each membrane preparation. The number of animals or the number of membrane preparations from which the release or enzyme data was derived is provided by the number in parenthesis. The ratios of the Na, Ca-phosphatase to the ouabain-sensitive (Na, K) Mg-ATPase (Na, K-ATPase) activity are compared in the last column. PTHx, parathyroidectomy; PTH, parathyroid hormone.

* $P < 0.01$.

‡ $P < 0.025$.

§ $P < 0.005$.

TABLE V
Influence of Inhibitors on the Phosphatase Activity

| | Na, Ca-dependent phosphatase activity | % Inhibition |
|------------------------|--|-----------------|
| | <i>nmol P_i/mg protein/min</i> | |
| PTHx + PTH | | |
| Control | 40.5 ± 2.7 | — |
| Ouabain (1 mM) | 39.2 ± 1.8 | 3 |
| Ethacrynic acid (1 mM) | 28.9 ± 3.3 | 29 |
| PTHx - PTH | | |
| Control | 24.4 ± 3.0 | — |
| Ethacrynic acid (1 mM) | 14.3 ± 2.2 | 41 |

The animals were prepared as described in text. Both groups were parathyroidectomized (PTHx) and given either parathyroid extract (PTH) (30 U) or diluent 4 h before sacrifice. The data represent the mean ± SEM of three separate membrane preparations.

tivity of the Na, Ca-dependent phosphatase. This response to parathyroid hormone was specific for the Na, Ca-dependent phosphatase, no stimulation being

TABLE VI
⁴⁵Ca Binding to Mucosal Cell Membrane

| Animal | Basal-lateral | | Brush border | |
|-----------------|----------------|-----------------|----------------|-----------------|
| | Ca | Ca + Na | Ca | Ca + Na |
| A. | | | | |
| PTHx-sham | 4,941 ± 183 | 24,892 ± 289 | 3,472 ± 203 | 10,922 ± 186 |
| PTHx | 4,285 ± 192 | 16,163 ± 223 | 3,514 ± 176 | 10,090 ± 254 |
| P value | NS | <0.05 | NS | NS |
| B. | | | | |
| Intact | 3,820 ± 220 | 18,230 ± 320 | | |
| Intact, trypsin | 3,683 ± 202 | 12,508 ± 173 | | |
| P value | <0.05 | <0.05 | | |

In series A, animals were prepared 24 h use by either parathyroidectomy (PTHx) or sham operation (PTHx-sham). In series B, the animals were maintained for 3 days on a low calcium diet. The binding of ⁴⁵Ca was determined by the competitive binding assays employing the cation-exchange resin Chelex-100 equilibrated with 0.1 mM magnesium as described in text. The concentration of calcium was 0.04 mM and the concentration of sodium, 120 mM. In series B, the membranes were preincubated for 30 min in trypsin (0.1 mg/ml) or a boiled trypsin preparation. Each value represents the mean ± SEM of eight determinations from two preparations of animals.

observed for calcium-dependent phosphatase activity or (Na, K) Mg-ATPase activity (Table IV). When expressed per unit (Na, K) Mg-ATPase or per milligram protein, the Na, Ca-dependent phosphatase activity was increased twofold in the presence of the hormone compared to the enzyme activity of the parathyroidectomized animals.

Of the classic inhibitors of the (Na, K) Mg ATPase activity, ouabain and ethacrynic acid, only ethacrynic acid inhibited the Na, Ca-dependent phosphatase (Table V). Ouabain, which does not influence calcium accumulation by the everted rat intestine (4) or the release of calcium from the mucosal epithelial cell (3) had no effect on the enzyme activity. Ethacrynic acid inhibits intestinal calcium absorption (4) but does not influence significantly the fractional rate of calcium release from the mucosal cell. However, the diuretic at a concentration of 1 mM inhibited 41% of the Na, Ca-dependent phosphatase activity in the 24-h parathyroidectomized animal (Table V). In the presence of parathyroid hormone, total Na, Ca-dependent phosphatase activity increases; however, the percent of the total activity by ethacrynic acid decreases relative to that of the parathyroid hormone-deficient animal. Thus ethacrynic acid is inhibiting a fraction of Na, Ca-dependent phosphatase activity which is not responsive to parathyroid hormone stimulation.

The apparent specificity and affinity of the enzyme for calcium at low calcium concentrations suggested that it would be possible to measure directly the interaction of calcium with the enzyme. By utilizing the principles of the methodology described by Wasserman and Taylor for identifying a calcium-binding protein of the mucosal epithelial cell (11), specific binding of ⁴⁵Ca to the basal-lateral membrane fragments could be demonstrated. These studies were carried out in buffer containing 0.1 mM magnesium, a concentration 25 times that of the concentration of calcium. This binding was enhanced in the presence of 120 mM sodium. The increment in calcium binding resulting from the addition of sodium was greatest in the basal-lateral membrane fraction. In the absence of parathyroid hormone, sodium-dependent binding of ⁴⁵Ca was significantly reduced in only the basal-lateral membrane preparation. This reduction was proportional to the reduction in Na, Ca-dependent phosphatase activity observed in the same membrane preparation. The sodium-dependent binding of the brush border was not reduced in the absence of parathyroid hormone and reflects in part displacement of the isotope from the resin by sodium. Preincubation of the membrane preparation with trypsin for 30 min reduced the binding of ⁴⁵Ca (Table VI).

DISCUSSION

Intestinal calcium absorption is ultimately dependent on the transport of calcium across the base of the mucosal epithelial cell. This step is dependent on sodium, is stimulated by parathyroid hormone, and requires the movement of calcium against both a concentration and electrical potential gradient (1-3, 13-16). In identifying the enzyme complex mediating this important step in the calcium absorption process a number of constraints could be established to which the enzyme should theoretically conform. Initially a model of cation translocation across the plasma membrane was assumed which was similar to that described for the movement of calcium across membranes of other tissues (17, 18). Such a model would dictate that the enzyme be a phosphatase, hydrolyzing ATP as a substrate and the driving force of the transport process. The activity of this enzyme would be dependent on calcium. In addition, a dependence of the enzyme on sodium would also be predicted from the observed dependence on sodium of calcium efflux from the mucosal cell (3). In contrast to calcium efflux in muscle and nerve cells which is dependent on a sodium gradient across the membrane (19), calcium efflux from mucosal epithelial cells is not blocked by inhibitors of the sodium pump. The mucosal enzyme complex would be localized predominately in the basal-lateral membranes in order to effect the active transport of calcium across the intestine. Differentiation of the luminal surface of the intestinal epithelial cell into a morphologically distinct structure permits the fractionation of the cell membrane into two functionally distinct components, the brush border and the basal-lateral membranes. Isolation of basal-lateral membranes yields a membrane fraction which was enriched with respect to ouabain-sensitive (Na, K) Mg-ATPase activity relative to brush border and mitochondrial fractions. Although the three membrane fractions demonstrated calcium dependent-phosphatase activity, only the basal-lateral membrane fraction demonstrated enhancement of the calcium-dependent phosphatase by sodium at pH 7.4. It is, therefore, unlikely that the Na, Ca-dependent phosphatase activity of the basal-lateral membrane fraction is a contaminant from mitochondrial or brush border membrane elements.

The cation dependency of the enzyme demonstrated a number of interesting features. Unlike the previously reported calcium-dependent phosphatases implicated in membrane transport of calcium (20, 21), magnesium was not required for activity. Optimum activity was achieved in the absence of magnesium at a calcium concentration of 0.04 mM and a sodium concentration of 200 mM. Since the enzyme activity was essentially independent of magnesium, this cation would be added to the reaction buffer at 10-100 times the concentration of

calcium. By so doing, nonspecific divalent cation-dependent activity could be determined. The increase in activity obtained by the addition of calcium and calcium plus sodium to the magnesium-stimulated system can therefore be attributed to specific Ca-dependent phosphatase activity. Under these conditions, significant and specific Na, Ca-dependent activity was demonstrated at 0.004 mM calcium. Intracellular calcium concentration has been estimated to be of the order of magnitude of 10^{-4} M and for cytosol, 10^{-5} or less (22-24). The optimum sodium concentration for this phosphatase was found to be 200 mM and, therefore, comparable to the extracellular sodium concentration. These observations suggest a mechanism analogous to the (Na, K) Mg-ATPase model in which the enzyme is activated by the intracellular sodium and extracellular potassium (25). Accordingly intracellular calcium and extracellular sodium would be required for the activation of the phosphatase and ultimately the translocation of calcium across the plasma membrane. The analogy may be extended with respect to the preferred substrate. Although a limited number of phosphate esters have been examined, adenosine triphosphate appears to be the preferred substrate.

The alkaline pH optimum for the enzyme is characteristic of intestinal alkaline phosphatases. These enzymes are now considered to represent a heterogeneous group of enzymes (26, 27). However, the physiologic function of these enzymes which are so abundant in the intestine have not been defined. It is intriguing to speculate as to their role in intestinal transport. There is some evidence to suggest that an alkaline phosphatase may mediate phosphate transport across the intestine (28). In addition, it has also been proposed that a calcium-dependent alkaline phosphatase associated with the brush border may also be involved in calcium transfer across this membrane (20).

In an attempt to further implicate the Na, Ca-dependent phosphatase in intestinal calcium transport, two physiologic alterations in intestinal calcium transport were examined. The first was the influence of age on the transport process since after the 4th week of age, active intestinal calcium absorption decreases markedly (13, 14). Similarly, the rate of ^{45}Ca release from the intact mucosal epithelial cell is also decreased by the age of 6 wk. Again the movement of calcium across the base of the mucosal epithelial cell would appear to be the limiting determinant of calcium transport under these conditions. As anticipated from these observations, the Na, Ca-dependent phosphatase activity was also diminished at 6 wk when expressed per milligram protein or per unit of (Na, K) Mg-ATPase activity. Thus the fall in activity appears to be specific for the Na, Ca-dependent phosphatase, affecting to a much lesser ex-

tent the activity of the ouabain-sensitive (Na, K) Mg-ATPase and basal calcium- or magnesium-dependent phosphatase.

A second important physiologic determinant of intestinal calcium absorption and transport across the basal-lateral membrane of the mucosal cell is parathyroid hormone. As noted previously (3), the intestinal response to parathyroid hormone, rather than being direct, may be mediated through the action of the hormone on the metabolism of vitamin D. Parathyroidectomy significantly reduced the rate of ^{45}Ca release from the mucosal epithelial cell as well as the Na, Ca-dependent phosphatase activity isolated from similar animals. The administration of parathyroid hormone to the parathyroidectomized animal restored both the rate of ^{45}Ca release and the Na, Ca-dependent phosphatase activity. The changes in the enzyme activity were again specific for the Na, Ca-dependent phosphatase since no changes in the (Na, K) Mg-ATPase activity were demonstrated. The changes in the release of ^{45}Ca in response to parathyroid hormone again paralleled the changes in net calcium transport by the intact intestine described by others under similar experimental conditions (14, 15, 29). The stimulation of Na, Ca-dependent phosphatase activity was, however, only twofold which suggested that perhaps a significant proportion of the activity measured may not be hormone dependent. This possibility was further supported by the study of the effect of ethacrynic acid on the enzyme. Administration of parathyroid hormone to parathyroidectomized rats increased total Na, Ca-dependent phosphatase activity but did not increase the activity of the ethacrynic acid-sensitive component of the enzyme activity. Thus a portion of the Na, Ca-dependent phosphatase activity (the ethacrynic acid-sensitive component) is not hormone dependent. Since ethacrynic acid does not influence the fractional rate of ^{45}Ca release from the mucosal epithelial cell (3), this ethacrynic acid-sensitive component of the enzyme would not be expected to participate in the transport mechanism. The inhibition of net calcium transport across the intact intestine by ethacrynic acid (4) must, therefore, be mediated through the inhibition of calcium uptake across the brush border.

In addition to the demonstration of parathyroid hormone-dependent enzyme activity, calcium binding to the plasma membrane preparation was also demonstrated to be parathyroid hormone dependent. This calcium binding activity of the membrane shares several important characteristics with the enzyme activity. In addition to the hormone dependence, the activities of both enzyme and calcium binding (a) are enhanced by sodium, (b) are localized predominantly in the basal-lateral membrane fraction, (c) are active at 0.05 mM calcium in the presence of 0.1 mM magnesium, and

(d) are inactivated by trypsin. In contrast, the binding of calcium by the calcium-binding protein identified by Wasserman is not enhanced by sodium (30). It is intriguing to speculate as to the association between the calcium binding by the membrane observed under these conditions and the calcium binding observed by electron microscopy along the cytoplasmic surface of the lateral membranes of the intestinal mucosal cell (31). At present these observations are consistent with parathyroid hormone directly or indirectly increasing the activity or synthesis of the Na, Ca-dependent enzyme. The consistent correlation under a variety of experimental conditions between the observed rate of ^{45}Ca release from the intestinal epithelial cell and the activity of a Na, Ca-dependent phosphatase provides indirect evidence for the participation of this alkaline phosphatase in the energy-dependent, rate-limiting step in the transport of calcium across the intestine.

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