Identification and isolation of the phosphorylated intermediate of the calcium pump in rat intestinal basolateral membranes

Renata WAJSMAN, Julian R. F. WALTERS* and Milton M. WEISER

Division of Gastroenterology and Nutrition, State University of New York at Buffalo, Buffalo General Hospital, 100 High Street, Buffalo, NY 14203, U.S.A.

Transport of Ca^{2+} by the ATP-dependent Ca^{2+} pump has been demonstrated previously in rat intestinal basolateral-membrane vesicles. To identify the Ca^{2+} -pump protein, duodenal basolateral membranes were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of Ca^{2+} and La^{3+} , under conditions conducive for maximal formation of the phosphorylated intermediate of the Ca^{2+} pump. Four major phosphoprotein bands were seen on autoradiograms of acidic SDS/polyacrylamide gels; the properties of a phosphoprotein (pp) at 130 kDa (pp130) were consistent with those expected for the plasma-membrane Ca^{2+} pump. This phosphoprotein was markedly enhanced by La^{3+} , exhibited the characteristics of an acyl-phosphate bond, was preferentially phosphorylated from ATP and inhibited by micromolar concentrations of vanadate. Another phosphoprotein of 115 kDa possibly represented the endoplasmic reticulum Ca^{2+} pump or a fragment of pp130. Other phosphoproteins of 75 and 95 kDa were predominantly expressions of alkaline phosphatase. Formation of pp130 was highest in duodenal basolateral-membrane preparations when compared with those of jejunum and ileum or other subcellular fractions. A similar correlation between Ca^{2+} -pump activity and pp130 formation was not found in membranes from villus-tip and crypt cells or in vitamin D-deficient animals. pp130 was isolated as a single phosphoprotein by calmodulin-affinity chromatography. We conclude that pp130 represents the phosphorylated intermediate of the rat intestinal basolateral-membrane Ca^{2+} pump, which can be separated from other phosphoproteins using its properties as a calmodulin-binding protein.

INTRODUCTION

Ca^{2+} transport across the basolateral membrane of the intestinal epithelial cell is the energy-requiring step in the absorption of dietary Ca^{2+} by the transcellular route [1, 2]. Studies of Ca^{2+} uptake into inside-out basolateral-membrane vesicles have demonstrated an ATP-dependent, calmodulin-stimulated Ca^{2+} pump which is capable of transporting Ca^{2+} against the 10000-fold concentration gradient at the basolateral membrane and maintaining a submicromolar free Ca^{2+} concentration in the cytoplasm [3–9]. These properties are similar to those of the plasma-membrane Ca^{2+} pumps found in other cells [10]. Important additional properties of the intestinal Ca^{2+} pump in keeping with its role in Ca^{2+} absorption are that the activity varies according to location (being greater in duodenal than ileal cells and in villus-tip than crypt), and may be dependent on the vitamin D status of the animal [6, 7].

Studies of the partial reactions of other plasma-membrane Ca^{2+} pumps have shown a Ca^{2+} -dependent transfer of the γ -phosphate from ATP to the catalytic part of the enzyme, which results in the formation of a phosphorylated intermediate of the enzyme (E_1P) [10, 11]. This has an acid-stable, hydroxylamine-sensitive phospho-aspartyl bond, and its steady-state level is enhanced by the addition of La^{3+} , which blocks the $\text{E}_1\text{P} \rightarrow \text{E}_2\text{P}$ transition [12]. Demonstration of this phosphoprotein (pp), with a molecular mass of between 130 and 150 kDa and appropriate properties, has led to the identification and subsequent purification of the Ca^{2+}

pumps from the erythrocyte [13], heart sarcolemma [14], brain [15] and Ehrlich ascites carcinoma cells [16].

Unlike these cells, epithelial cells from the intestine transport large amounts of Ca^{2+} from dietary sources and are subject to a variety of physiological controls that might be expected to affect Ca^{2+} -pump properties [17]. This paper establishes the presence of a 130 kDa phosphorylated intermediate of the intestinal Ca^{2+} pump in rat duodenal basolateral membrane and correlates the subcellular and regional distribution with pump activity. By exploiting the binding of the Ca^{2+} -pump protein to calmodulin in the presence of Ca^{2+} , we were able to use calmodulin-affinity chromatography to purify this protein.

EXPERIMENTAL PROCEDURES

Materials

Chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) unless specified. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was supplied by ICN Radiochemicals (Irvine, CA, U.S.A.); electrophoresis materials, protein assay materials and calmodulin-agarose were from Bio-Rad (Richmond, CA, U.S.A.). Animals were from Holtzman (Madison, WI, U.S.A.).

Membrane preparation

The proximal 10–15 cm of intestine (duodenum and upper jejunum) was removed from 5–7-week-old male rats which had been fasted overnight. The middle

Abbreviations used: pp, phosphoprotein; PAGE, polyacrylamide-gel electrophoresis.

* To whom correspondence should be addressed at: Gastroenterology Unit, 18th Floor, Guy's Tower, London SE1 9RT, U.K.

15 cm of the small intestine (jejunum) and the distal 15 cm of the small intestine (ileum) were also prepared from some animals. Membrane fractions enriched approx. 10-fold for Na^+/K^+ -ATPase activity were prepared from isolated villus and crypt cells as previously described [7]. For subcellular distribution experiments, crude brush-border-enriched fractions were prepared from a 45–50 g/dl sorbitol density fraction, and crude microsomes from the 105 000 $\text{g} \times 60$ min pellet. Some rats were fed a vitamin D-deficient diet [7]. To increase the protein yield for purification experiments, basolateral membranes were prepared from cells isolated by the method of Weiser [18] from 30 cm of proximal intestine. ATP-dependent Ca^{2+} uptake was performed on representative basolateral membrane vesicles at 0.5 μM -free Ca^{2+} to confirm the presence of an active Ca^{2+} pump; protein and other enzyme activity measurements were also as previously described [7].

Phosphorylation of membrane proteins

Phosphorylation experiments were performed with membranes that were freshly isolated or which had been frozen at -70°C for no more than 96 h with soya-bean trypsin inhibitor (0.05%), phenylmethanesulphonyl fluoride (0.2 mM), and dithiothreitol (2 mM). Membranes (50–200 μg of protein) were incubated on ice in a total volume of 200 μl containing 100 mM-KCl/30 mM-Hepes (pH 7.0)/50 μM - CaCl_2 /100 μM - LaCl_3 where indicated. Bovine serum albumin (75 μg) was usually added to aid protein precipitation. Other components were present, as indicated in the Figure legends. To test the effect of vanadate on phosphoenzyme formation, basolateral membranes were preincubated with 15 μM -sodium orthovanadate in the presence of 1.5 mM- MgCl_2 and 100 mM-KCl for 5 min. Phosphorylation was started by the addition of [γ - ^{32}P]ATP (specific radioactivity 5–20 Ci/mmol), final concentration 2–6 μM , under vigorous vortexing. The reaction was stopped after 60 s by the addition of 500 μl of 7% (v/v) trichloroacetic acid containing 1 mM-ATP and 25 mM-inorganic phosphate, and the samples were washed three times with the above solution. Some pellets were treated with hydroxylamine (0.8 M-hydroxylamine/HCl/0.08 M-sodium acetate, pH 5.6) for 10 min at 20°C . The washed precipitates were dissolved at room temperature in the electrophoresis sample buffer containing 0.15 M-Tris/phosphate (pH 6.8), 11% (v/v) glycerol, 4% (w/v) SDS, 4% (v/v) β -mercaptoethanol and 0.0125% (w/v) Bromophenol Blue. Aliquots were taken for ^{32}P determination by liquid-scintillation counting.

Gel electrophoresis

To preserve the alkali-sensitive acyl-phosphate bond, SDS/polyacrylamide-gel electrophoresis (PAGE) was carried out under mildly acidic conditions according to Sarkadi *et al.* [19], except that the running gel was modified to contain 5% (w/v) acrylamide with 0.03% (w/v) ammonium persulphate and 0.025% (v/v) N,N,N',N' -tetramethylethylenediamine for polymerization. Autoradiography of the dried gels was performed with 12–72 h exposure at -70°C using Kodak X-omatic film. The molecular masses of the membrane proteins were estimated by the mobilities of [^{14}C]methylated protein standards run on the same gel (Sigma molecular-mass markers, 30 000–200 000). Densitometric tracings were made of selected lanes of the autoradiograms.

Calmodulin-affinity chromatography

This was based upon the procedure described for the erythrocyte Ca^{2+} pump [13]. Basolateral-membrane protein (70–100 mg), frozen overnight in Buffer A (300 mM-KCl / 20 mM-Hepes (pH 7.4) / 2 mM-dithiothreitol / 100 μM -phenylmethanesulphonyl fluoride / 1 mM- MgCl_2 / 100 μM - CaCl_2) with 0.5 mg of soya-bean trypsin inhibitor/ml, was solubilized with 1 mg of Triton X-100/mg of protein in Buffer A and centrifuged at 105 000 g for 20 min at 4°C . Phosphatidylcholine (a sonicated suspension of Sigma type IX-E) and EGTA were added to the supernatant to give final concentrations of 0.3 mg/ml and 1 mM respectively. To deplete endogenous calmodulin, the procedure of Wetzker *et al.* [16] was followed, using batch treatment with DEAE-Sephadex ion-exchange resin. After addition to the supernatant of CaCl_2 (final concentration 1.5 mM), the protein was applied to a 1 ml calmodulin-agarose affinity column which had been equilibrated with Buffer B (same as Buffer A but also containing 5 mg of Triton X-100/ml and 0.3 mg of phosphatidylcholine/ml). The column was then washed with 20 ml of Buffer C (same composition as Buffer B but with 100 mM-KCl). Additional washes of 40 ml were done with Buffer D (same as Buffer C but with 1 mg of Triton X-100/ml and 0.06 mg of phosphatidylcholine/ml). Protein bound to the affinity column was eluted with Buffer E (where 1 mM-EGTA replaced CaCl_2 in Buffer D). The eluate was collected in sequential 0.7 ml fractions, or pooled and concentrated in an ultrafiltration chamber with a PM-30 filter (Amicon, Lexington, MA, U.S.A.). Eluates were then phosphorylated using the same protocol as for basolateral membranes except that the total volume was increased up to 700 μl and the KCl concentration was 100 mM. Free Ca^{2+} was adjusted to 50 μM , and La^{3+} to 100 μM , assuming complete chelation of La^{3+} by EGTA.

RESULTS

Phosphorylation pattern

The conditions of phosphorylation were optimized for the formation of an acid-stable, hydroxylamine-sensitive, acyl-phosphate bond. As shown in studies with other tissues [12,19,20], La^{3+} was included to stabilize the phosphorylated intermediate of the Ca^{2+} pump. The pattern of ^{32}P incorporation into proteins from duodenal basolateral membrane is shown in the autoradiogram in Fig. 1. A band with approximate molecular mass of 130 kDa (pp130) was clearly seen in the presence of Ca^{2+} and La^{3+} (lane 2). Without La^{3+} (lane 1), the intensity of this band was greatly reduced, presumably from dephosphorylation. No band was seen in the presence of EGTA (lane 3) and, in keeping with an acyl-phosphate bond [10], the band was completely abolished when the pellets of proteins phosphorylated in the presence of Ca^{2+} and La^{3+} were treated with hydroxylamine (lane 4) or boiled (lane 5). Formation of pp130 was unaffected by the addition of Mg^{2+} , in either millimolar or micromolar concentrations. Phosphorylation reactions stopped after 15 s showed a similar level of pp130 as after 60 s, and this persisted for at least 3 min. pp130 could not be reliably demonstrated in preparations frozen overnight, unless they were stored at -70°C with proteolysis inhibitors.

Another band at 115 kDa (pp115) was also phosphorylated in the presence of Ca^{2+} and enhanced by

Fig. 1. Phosphorylation pattern of rat duodenal basolateral membranes

Basolateral membranes were phosphorylated by [γ - ^{32}P]-ATP under various conditions. Lane 1, + Ca^{2+} (50 μM); lane 2, + Ca^{2+} (50 μM) + La^{3+} (100 μM); lane 3, + EGTA (0.5 mM); lane 4, as lane 2, phosphoprotein pellet treated with hydroxylamine; lane 5, as lane 2, SDS-dissolved samples boiled for 4 min. Other conditions were as described in the Experimental procedures section.

La^{3+} . Its presence was inconsistent and although it had acyl-phosphate characteristics, unlike pp130 it was phosphorylated in the presence of excess EGTA (lane 3). Another variable minor band at approx. 50 kDa was formed in a Ca^{2+} -dependant fashion (lanes 1 and 3) but was not increased by La^{3+} . In addition, major phosphoprotein bands were seen at 95 kDa and 70 kDa in the presence of Ca^{2+} but had no specific requirement for it (lane 3). They were unaffected by La^{3+} , hydroxylamine treatment or boiling (lanes 2, 4, and 5 respectively).

Analysis of densitometric tracings showed pp130 to comprise about 18% of the total phosphorylation of pp130, pp115, pp95 and pp70 in the presence of Ca^{2+} and La^{3+} (Table 1). Total incorporation of ^{32}P was 9.7 ± 2.6 pmol/mg of basolateral-membrane protein (mean $\pm$ S.E.M., $n = 8$). Consequently the amount of pp130 was about 1.8 pmol/mg of protein.

Fig. 2. Substrate specificity and effects of vanadate on phosphorylation of duodenal basolateral membranes

Membranes in all lanes were phosphorylated with Ca^{2+} and La^{2+} . Lane 1, no addition; lane 2, + β -glycerophosphate (5 mM); lane 3, + p -nitrophenyl phosphate (5 mM); lane 4, + AMP (5 mM); lane 5, + orthovanadate (15 μM) with preincubation with Mg^{2+} and K^+ .

Substrate specificity and inhibitors

Substrate specificities of the phosphoproteins are displayed in Fig. 2. Complete inhibition of labelling of pp70 occurred with addition of β -glycerophosphate, p -nitrophenyl phosphate or AMP (lanes, 2, 3 and 4 respectively), indicating phosphorylation from these non-radioactive phosphate substrates and no preference for [γ - ^{32}P]ATP. Phosphoprotein 95 was also inhibited, except for a small residue (see the Discussion section). On the other hand, despite the excess of other phosphate substrates, pp130 was phosphorylated only by [γ - ^{32}P]ATP. Analysis of the densitometry tracings (Table 1) and ^{32}P counts showed a reduction of total phosphorylation to about 50% by β -glycerophosphate, which was almost entirely due to the reduction in pp70 and pp95.

Preincubation of basolateral-membrane protein with vanadate in the presence of Mg^{2+} and KCl resulted in decreased formation of pp130, and pp115, with occasionally some inhibition of pp70 (Fig. 2, lane 5 and Table 1). As expected, there was no change in pattern of phosphoenzyme formation upon addition of calmodulin (1–2 μM) to the phosphorylation medium; intestinal basolateral-membrane preparations are known to contain large amounts of endogenous calmodulin [9].

Table 1. Phosphorylation of intestinal basolateral-membrane proteins: effects of β -glycerophosphate and vanadate

Phosphorylation from [γ - ^{32}P]ATP was performed in the presence of Ca^{2+} and La^{3+} , and after addition of 5 mM- β -glycerophosphate (βGP) or 15 μM -orthovanadate (Van). Results are expressed as mean $\pm$ S.E.M. and were calculated from densitometric tracings that were standardized so that the mean total density of the phosphoprotein bands at 70, 95, 115 and 130 kDa was 100% in the presence of Ca^{2+} and La^{3+} alone. Total phosphorylation in the presence of β -glycerophosphate and orthovanadate, and the individual bands, is also expressed as a percentage of the mean total phosphorylation without inhibitors.

Conditions	<i>n</i>	Total	Phosphoprotein (kDa)...	Fraction of total phosphoprotein (%)			
				70	95	115	130
$\text{Ca}^{2+}/\text{La}^{3+}$	8	100 ± 14		14 ± 3	51 ± 4	18 ± 2	18 ± 3
$\text{Ca}^{2+}/\text{La}^{3+}/\beta\text{GP}$	3	44 ± 8		1 ± 0	19 ± 1	11 ± 0	14 ± 2
$\text{Ca}^{2+}/\text{La}^{3+}/\text{Van}$	3	60 ± 2		11 ± 4	46 ± 2	3 ± 2	1 ± 0

Fig. 3. Phosphorylation pattern in different subcellular fractions and preparations of membranes

All fractions contained 50 μ g of protein and were phosphorylated in the presence of Ca^{2+} and La^{3+} . Basolateral membranes were prepared from different regions: (1) duodenum, (2) jejunum and (3) ileum. Duodenal fractions were compared: (4) homogenate, (5) microsomes, (6) brush-border-enriched membranes, and basolateral membranes prepared from (7) villus-tip cells, (8) crypt cells, and (9) scrapings of mucosa. Duodenal basolateral membranes were compared in animals fed (10) a vitamin D-sufficient diet and (11) a vitamin D-deficient diet.

Calmidazolium, a calmodulin inhibitor, did not inhibit phosphoenzyme formation at micromolar concentrations. Quercetin (10 μ M), which inhibits active Ca^{2+} uptake in duodenal basolateral-membrane vesicles [21], had no effect on phosphoenzyme formation (results not shown).

Regional and subcellular phosphorylation patterns

Distribution of the phosphoproteins in different preparations is shown in Fig. 3. Compared with the duodenum (lane 1), basolateral membranes prepared from the mid-jejunum (lane 2) and terminal ileum (lane 3) showed phosphoprotein bands, including pp130, of lower density. In duodenal homogenates (lane 4), microsomal fractions (lane 5) and brush-border membranes (lane 6), pp130 was only weakly phosphorylated. Despite differences in ATP-dependent Ca^{2+} uptake, basolateral membranes prepared from duodenal villus-tip cells had similar levels of pp130 to those from crypt cells (lanes 7 and 8). Basolateral membranes prepared from scrapings of duodenal mucosa [23], though similarly enriched for Na^+/K^+ -ATPase as those prepared from isolated cells, did not have ATP-dependent Ca^{2+} uptake [23]; in these membranes (lane 9), mean pp130 was reduced to 35% of that seen in the membranes of isolated cells. Basolateral-membrane fractions prepared from animals fed a vitamin D-deficient diet were shown to possess lower vesicular active Ca^{2+} transport [7], but appeared to have the same level of pp130 as their vitamin D-sufficient controls (lanes 10 and 11).

Purification

Solubilization of basolateral membranes with Triton X-100 and DEAE-cellulose treatment resulted in reduction of total protein to about 50%. Patterns of phosphorylation of proteins at each step are seen in Fig. 4. pp130, 95, 70, and 50 were clearly demonstrable

Fig. 4. Phosphorylation pattern during steps in the purification of the duodenal basolateral-membranes Ca^{2+} pump

All fractions were phosphorylated with Ca^{2+} and La^{3+} . Lane 1, original membranes; lane 2, Triton-solubilized membranes; lane 3, proteins after treatment with DEAE-cellulose; lane 4, proteins unbound to calmodulin-affinity column; lane 5, end of wash with Ca^{2+} -containing buffer; lane 6, pooled EGTA eluate from calmodulin-affinity column; lane 7, as lane 6 + β -glycerophosphate (5 mM).

in fractions after Triton solubilization (lane 2) and after DEAE-cellulose treatment (lane 3). Lane 4 indicates that some pp130 was included in the proteins that did not bind to the column. Samples obtained at the end of the Ca^{2+} -containing wash showed an absence of phosphoproteins (lane 5). After changing to the EGTA-containing buffer to elute Ca^{2+} -dependent calmodulin-binding proteins, sequential fractions of the eluate were phosphorylated and showed only one phosphoprotein at 130 kDa which was maximal at 3 ml of eluate. pp130 was also shown in pooled, concentrated eluate in the presence of Ca^{2+} and La^{3+} (lane 6) and was not inhibited when phosphorylated in the presence of 5 mM- β -glycerophosphate (lane 7).

DISCUSSION

The ATP-dependent Ca^{2+} pump comprises only a minor fraction (0.1–0.2%) of the plasma membrane proteins [10], which makes identification and purification a difficult task. In this study of rat duodenal basolateral membranes, previously demonstrated to have Ca^{2+} -pump activity [7], [γ - ^{32}P]ATP was used under optimal conditions to identify the phosphorylated intermediate of the Ca^{2+} pump by acidic SDS/PAGE. This was then isolated as a single phosphoprotein by calmodulin-affinity chromatography.

The phosphoprotein band identified at 130 kDa appears to be the phosphorylated intermediate of the ATP-dependent Ca^{2+} pump for the following reasons: (i) the steady-state phosphorylation of pp130 increased significantly in the presence of La^{3+} ions and was inhibited by EGTA, as has been described for other plasma-membrane Ca^{2+} pumps [12,19,20]; (ii) it was sensitive to hydroxylamine, a characteristic of the acyl-phosphate bond at the active site of ion-motive ATPase [10,11]; (iii) the molecular mass corresponded to that of plasma-membrane Ca^{2+} pumps found in other tissues [13,15,20,24]; (iv) it had a specific requirement for ATP compared with β -glycerophosphate, *p*-nitrophenyl phosphate or AMP as substrates; (v) it was inhibited by orthovanadate in the

presence of Mg^{2+} and K^+ [25]; (vi) it bound calmodulin in a Ca^{2+} -dependent manner, as shown by its elution from a calmodulin-affinity column as a single phosphoprotein. This phosphoprotein is unlikely to represent the phosphorylated intermediate of the Na^+/K^+ -ATPase, which has a molecular mass of around 100 kDa [26], or the endoplasmic reticulum Ca^{2+} -ATPase, which has a molecular mass of about 115 kDa [27] and does not have enhanced phosphorylation in the presence of La^{3+} .

Although the band at 115 kDa appeared to have similar acyl-phosphate characteristics to pp130, its presence was variable. Although these basolateral-membrane-enriched fractions are not completely depleted of endoplasmic reticulum marker-enzyme activities [7], it is difficult to reconcile the lack of effect of EGTA on this band with an identity as the endoplasmic reticulum Ca^{2+} -ATPase. This protein was not eluted from the calmodulin-affinity column, but it could possibly represent a fragment of the 130 kDa protein unable to bind to calmodulin. A non-covalent association of a 35 kDa and a 80–90 kDa proteolytic fragment of the erythrocyte pump has also been observed in an acidic gel-electrophoresis system [19]. The phosphorylation of the band seen around 50 kDa was enhanced with Ca^{2+} but was inhibited by La^{3+} . It may also be a proteolytic fragment of the Ca^{2+} pump or perhaps be a product of a protein kinase phosphorylation.

The phosphoprotein bands at 95 kDa and at 70 kDa display properties different from those described for the Ca^{2+} pump. The lack of dependency on Ca^{2+} , the competition for phosphorylation by β -glycerophosphate, *p*-nitrophenyl phosphate and AMP, and the insensitivity to hydroxylamine and La^{3+} , are similar characteristics observed for phosphoproteins in kidney membranes at 85 kDa [20] and intestinal scrapings at 64 kDa and 84 kDa [28], which were believed to be alkaline phosphatase. Rat intestinal alkaline phosphatase appears to be heterogeneous on SDS/PAGE, running as various isoforms at 92, 79 and 64 kDa [29]. Further evidence for these bands being alkaline phosphatase is their presence in brush-border-enriched fractions and their reduced intensity in ileal preparations. In the presence of β -glycerophosphate, a residual band remained at about 95 kDa and was not enhanced by La^{3+} , exhibited hydroxylamine sensitivity, and did not bind to the calmodulin-affinity column. Similar observations were made about a 100 kDa band in kidney basolateral membrane [20] and carcinoma cells [24]; this band may represent Na^+/K^+ -ATPase, despite the presence of conditions unfavourable to its phosphorylation (ouabain or absence of Na^+), or alternatively may be a non-calmodulin binding fragment of pp130.

Some other properties of pp130 included no requirement for Mg^{2+} at the phosphoprotein-intermediate formation step. Phosphorylation rapidly reached a level that remained stable for at least 3 min, being unaffected by quercetin, endogenous calmodulin, or calmodulin antagonists. These later observations are not surprising in view of the high endogenous concentrations of calmodulin in intestinal basolateral-membrane fractions and the relative difficulty in removing them [9]. Micromolar concentrations of orthovanadate decreased the amount of pp130 and also inhibited intestinal basolateral-membrane vesicular Ca^{2+} transport [7], but were shown not to affect Ca^{2+} -ATPase activity [30]. A similar dissociation of properties has been demonstrated in liver plasma

membranes [31]. Formation of the phosphoprotein appeared to be sensitive to proteolysis, as has also been described for vesicular Ca^{2+} -transport activity [22]. However, no proteolytic fragments could be clearly identified as forming phosphorylated intermediates or binding to the calmodulin column, as might have been expected from findings with the red cell Ca^{2+} pump [19].

Phosphorylation of basolateral membranes obtained from scrapings of rat intestinal mucosa was described by De Jonge *et al.* [28]. They showed bands at 64 and 84 kDa which displayed characteristics similar to our pp70 and pp95, and were also identified as being consistent with alkaline phosphatase. However, they did not demonstrate a phosphoprotein at 130 kDa with the expected properties, but showed a Ca^{2+} -dependent phosphoprotein at 115 kDa as a poorly resolved shoulder of the major Ca^{2+} -insensitive peak of 84 kDa. Perhaps this phosphoprotein corresponded to the 115 kDa protein seen in our gels, or was a proteolytic fragment of the 130 kDa protein. The absence of La^{3+} and the strongly acidic conditions prevented maximal steady-state levels or good resolution of the phosphoprotein. Furthermore, in our experiments, when scrapings of mucosa were used, the amount of pp130 was greatly reduced and we have been unable to demonstrate vesicular Ca^{2+} uptake with these preparations of membrane fractions [23].

The regional distribution of the Ca^{2+} -pump phosphoprotein was studied to see whether it correlated with vesicular Ca^{2+} -transport. In the jejunum and ileum, which showed reduced Ca^{2+} -uptake [30], decreased formation of pp130 was seen. However, no differences in phosphoprotein levels were detected in basolateral membranes from duodenal villus-tip and crypt cells, and a correlation between vesicular transport activity and phosphoprotein formation was not observed in vitamin-D deficient animals. This suggests that the activity of the intestinal Ca^{2+} pump may also be regulated at other steps than the amount of protein and its ability to form a phosphorylated intermediate.

We are grateful to Ms. Maud Charpin and Ms. Kathy Gniecko for their excellent technical assistance. This work was supported by USPHS Grants DK-35015 (to J.R.F.W.) and DK-32336 (to M.M.W.), and by the Margaret Duffy and Robert Cameron Troup Fund for Cancer Research of Buffalo General Hospital. R.W. was a National Institutes of Health Research Trainee and J.R.F.W. was the recipient of an American Gastroenterological Association/Industry Research Scholarship Award.

REFERENCES

1. Bronner, F., Pansu, D. & Stein, W. D. (1986) *Am. J. Physiol.* **250**, G561–G569
2. Van Os, C. H. (1987) *Biochim. Biophys. Acta* **906**, 195–222
3. Nellans, N. H. & Popovitch, J. E. (1981) *J. Biol. Chem.* **256**, 9932–9936
4. Ghijsen, W. E. J. M., de Jong, M. D. & van Os, C. H. (1982) *Biochim. Biophys. Acta* **689**, 327–336
5. Hildmann, B., Schmidt, A. & Murer, H. (1982) *J. Membr. Biol.* **65**, 55–62
6. Ghijsen, W. E. J. M. & van Os, C. H. (1982) *Biochim. Biophys. Acta* **689**, 170–172
7. Walters, J. R. F. & Weiser, M. M. (1987) *Am. J. Physiol.* **252**, G170–G177

8. Van Corven, E. J. J. M., Roche, C. & van Os, C. H. (1985) *Biochim. Biophys. Acta* **820**, 274–282
9. Ghijsen, W. E. J. M., van Os, C. H., Heizmann, C. W. & Murer, H. (1986). *Am. J. Physiol.* **251**, G223–G229
10. Rega, A. F. & Garrahan, P. J. (1986) *The Ca²⁺ Pump of Plasma Membranes*, CRC Press, Boca Raton
11. Pedersen, P. L. & Carafoli, E. (1987) *Trends Biochem. Sci.* **12**, 146–150
12. Luterbacher, S. & Schatzmann, H. J. (1983) *Experientia* **39**, 311–312
13. Niggli, V., Penniston, J. T. & Carafoli, E. (1979) *J. Biol. Chem.* **254**, 9955–9958
14. Caroni, P. & Carafoli, E. (1981) *J. Biol. Chem.* **256**, 3263–3270
15. Hakim, G., Itano, T., Verma, A. K. & Penniston, J. T. (1982) *Biochem. J.* **207**, 225–231
16. Wetzker, R., Böhmer, F. D., Klinger, R., Muller, E., Hegewald, H., Scheven, M. & Grosse, R. (1986) *Biochim. Biophys. Acta* **854**, 117–123
17. Favus, M. J. (1985) *Am. J. Physiol.* **248**, G147–G157
18. Weiser, M. M. (1973) *J. Biol. Chem.* **248**, 2536–2541
19. Sarkadi, B., Enyedi, A., Földes-Papp, Z. & Gárdos, G. (1986) *J. Biol. Chem.* **261**, 9552–9557
20. De Smedt, H., Parys, J. B., Borghgraef, R. & Wuytack, F. (1983) *Biochim. Biophys. Acta* **728**, 409–418
21. Walters, J. R. F. (1987) *Clin. Res.* **35**, 415 A (abstr.)
22. Van Corven, E. J. J. M., de Jonge, M. D. & van Os, C. H. (1986) *Cell Calcium* **7**, 89–99
23. Walters, J. R. F., Horvath, P. J. & Weiser, M. M. (1984) in *Epithelial Calcium and Phosphate Transport: Molecular and Cellular Aspects* (Bronner, F. & Peterlik, M., eds.), pp. 187–192, Alan R. Liss, New York
24. Spitzer, E., Böhmer, F. D. & Grosse, R. (1983) *Biochim. Biophys. Acta* **728**, 50–58
25. Barrabin, H., Garrahan, P. J. & Rega, A. F. (1980) *Biochim. Biophys. Acta* **600**, 796–804
26. Harms, V. & Wright, E. M. (1980) *J. Membr. Biol.* **53**, 119–128
27. Heilmann, C., Spamer, C. & Gerok, W. (1984) *J. Biol. Chem.* **259**, 11139–11144
28. De Jonge, H. R., Ghijsen, W. E. J. M. & van Os, C. H. (1981) *Biochim. Biophys. Acta* **647**, 140–149
29. Malik, N. & Butterworth, P. J. (1976) *Biochim. Biophys. Acta* **446**, 105–114
30. Moy, T. C., Walters, J. R. F. & Weiser, M. M. (1986) *Biochem. Biophys. Res. Commun.* **141**, 979–985
31. Lin, S.-H. (1985) *J. Biol. Chem.* **260**, 10976–10980

Received 31 May 1988/29 July 1988; accepted 3 August 1988