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PHOSPHORYLATED INTERMEDIATES OF CA^{2+}-ATPASE AND ALKALINE PHOSPHATASE IN PLASMA MEMBRANES FROM RAT DUODENAL EPITHELIUM

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Purified basolateral membranes of rat duodenal epithelium were phosphorylated in the presence of [γ-^{32}P]ATP and 1 μM free Ca^{2+} under conditions favorable for phospho-intermediate formation. Analysis of ^{32}P-labeled membrane proteins on SDS-acrylamide gel electrophoresis revealed the presence of three Ca^{2+}-sensitive phosphoproteins with molecular weights of 64 000 (protein I), 84 000 (II) and 115 000 (III). Both proteins I and II were identified as phospho-intermediate forms of alkaline phosphatase on the basis of the following criteria: these phosphoproteins were resistant to hydroxylamine and alkaline treatment, phosphate incorporation was stimulated by Zn^{2+} but totally inhibited by excess β-glycerophosphate during phosphorylation. A similar labeling efficiency was obtained with inorganic ^{32}P instead of [γ-^{32}P]ATP. Protein III comigrated on SDS gels with the phosphorylated intermediate of Ca^{2+}-ATPase from rat heart sarcolemnal membranes and was identified as the intestinal form of Ca^{2+}-ATPase in view of the following observations: binding of ^{32}P to this protein was hydroxylamine sensitive and alkaline labile, which is in agreement with an acylphosphate character of an ATPase intermediate, phospho-intermediate formation was dependent on low free Ca^{2+} concentrations (1 μM) in the presence of 1 mM Mg^{2+}, phosphorylation was strongly inhibited in the presence of phenothiazines (0.1 mM chlorpromazine or trifluoperazine). The amount of ^{32}P bound to Ca^{2+}-ATPase in basolateral membranes was only 6% of the amount incorporated into ($Na^+ + K^+$)-ATPase. The turnover number of duodenal Ca^{2+}-ATPase estimated from this phosphorylation study is remarkably similar to the value for the red blood cell Ca^{2+}-ATPase. Phosphorylation experiments with purified leaky brush border membranes under similar conditions did not reveal a phospho-intermediate form of Ca^{2+}-ATPase as demonstrated in basolateral membranes. In contrast about 7-fold higher levels of ^{32}P in phosphoprotein bands I and II were found. The ^{32}P-labeling characteristics of these proteins were similar to those of alkaline phosphatase intermediates in the basolateral membrane. The results of this study are fully in support of an earlier study in which high affinity Ca^{2+}-ATPase was found exclusively in the basolateral membrane of duodenal epithelium.

Introduction

In a previous study, kinetic analysis of Ca^{2+}-induced ATP hydrolysis in plasma membranes from rat duodenum demonstrated the presence of both high and low affinity sites for Ca^{2+} in the brush border and the basolateral membrane of duodenal epithelial cells [1]. A detailed examination of the substrate specificity of the ATPase plus the effect of inhibitors led to the conclusion that a specific high affinity Ca^{2+}-ATPase ($K_m \sim 0.4$ μM), the enzymatic expression of a Ca^{2+} pump, is exclusively located in the basolateral membrane [2]. In contrast, the low

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulphate.

affinity site of the ATPase ($K_m \sim 60$ μM) in this membrane, as well as the Ca^{2+}-ATPase in the brush border could be ascribed to activation of alkaline phosphatase, an ecto-enzyme unequally distributed among both plasma membranes [2–4]. Apparently, the susceptibility of alkaline phosphatase to Ca^{2+} resulted from depletion of Zn^{2+} from the active center due to EDTA which is used during membrane isolation and to EGTA which is used as a Ca^{2+}-buffer in the Ca^{2+}-ATPase assay.

Assuming a close homology between duodenal Ca^{2+}-ATPase and the Ca^{2+} pump in red blood cells and muscle [5,6], the catalytic part of the enzyme (E) was expected to form a hydroxylamine-sensitive phospho-acyl bond with the terminal phosphate of ATP as part of the general reaction scheme:

$$E_1 \xrightarrow{ATP,Ca^{2+}} E_1P \xrightarrow{Mg^{2+}} E_2P \xrightarrow{H_2O} E_2 \rightarrow E_1 \text{ (Refs. 7–9)}$$

(ADP from E_1P; P_i from E_2P)

Ca^{2+} dependent membrane phosphorylation by [γ-^{32}P]ATP has been used as a tool to analyze the mechanism of the Ca^{2+}-ATPase reaction and to visualize the enzyme on SDS-acrylamide gels [7–10]. Applying this method to intestinal plasma membranes provides us with criteria to distinguish between a specific Ca^{2+}-ATPase and other (Ca^{2+} + Mg^{2+})-dependent ATP hydrolyzing reactions such as alkaline phosphatase [2], a Ca^{2+}-calmodulin dependent protein kinase acting in concert with a protein phosphatase [11] and actin-myosin ATPase [12]. The latter possibility is suggested by the association of actin and myosin with baso-lateral membranes of duodenal epithelium [13]. The results of this phosphorylation study are fully in support of the conclusion that high affinity Ca^{2+}-ATPase is exclusively located in the basolateral plasma membrane of duodenal epithelial cells.

Methods and Materials

In this study we preferred to use plasma membranes which were isolated in the presence of EDTA. This isolation procedure yields leaky membrane fragments and guarantees a better access to cation and phosphate binding sites on both the *cis* and *trans* side of the membrane [2]. Mucosal scrapings of the first 15 cm of the duodenum of male wistar rats (180 g) were homogenized in 5 mM EDTA with a loosely-fitting Dounce apparatus as described by Mircheff and Wright [14]. A crude basolateral membrane fraction was prepared according to Mircheff et al. [15] as described in more detail elsewhere [2,13]. In some experiments crude basolateral membranes were purified further by zonal electrophoresis on density gradients to remove residual contamination of smooth endoplasmic reticulum [13]. Leaky brush border membrane vesicles were prepared essentially according to Mircheff and Wright [14]. Instead of centrifugation on linear sorbitol gradients, nuclear material was removed by centrifugation in 60% sorbitol for 1 h at 200 000 $\times g$. Brush borders were collected as a band at the interface between 60 and 25% sorbitol. This fraction contained 33 ± 7% (n = 3) of the initial sucrase and had similar enrichment factors to those reported by Mircheff and Wright [14]. Contamination with succinate dehydrogenase was 0.3 ± 0.1% (n = 3) of the initial activity.

Calmodulin was purified from bovine brain following the procedure of Dedman et al. [16]. The protein (mol. et. 17 000) appeared electrophoretically pure and highly active as a phosphodiesterase activator tested as described by Davis and Daley [17].

Enzyme assays. Ca^{2+}-ATPase activities were assayed in a standard medium containing 100 mM KCl or NaCl, 5 mM $MgCl_2$, 3 mM ATP and 50 mM Tris-maleate buffer (pH 7.4) at 25°C [2]. Free Ca^{2+} concentrations were stabilized by EGTA buffering as described previously [2]. Sucrase, (Na^+ + K^+)-ATPase, succinate dehydrogenase, NADPH-cytochrome *c* reductase and protein were assayed as described before [13].

Phosphorylation and electrophoresis of membrane proteins. Phosphorylation of membranes (150 to 300 μg of protein) was carried out in ice for 30 s in a total volume of 0.06 ml containing 40 mM histidine-imidazole buffer (pH 7.3), 5 mM KCl, 0.24 mM EGTA, 20 μm [γ-^{32}P] ATP (specific activity: 4–10 Ci/mmol) and varying concentrations of $MgCl_2$ and $CaCl_2$. Calculations of free Ca^{2+} concentrations were corrected for temperature according to Scharff [18]. Free Zn^{2+} concentrations were also buffered with EGTA assuming a binding ratio of total Zn^{2+} to total EGTA of unity [2]. Phosphorylation of (Na^+ + K^+)-ATPase was achieved by replacing KCl with 50 mM NaCl. In some experiments, ^{32}P-labeled inorganic

phosphate of similar specific activity was substituted for [γ-^{32}P]ATP. After incubation, membrane protein was precipitated with 0.2 ml ice-cold 3 M perchloric acid containing 2.5 mM ATP and 5 mM Na_2HPO_4. Precipitates were solubilized at room temperature in 0.08 ml solution containing 0.25 M sucrose, 20 mM NaH_2PO_4 (pH 2.4), 50 mM dithiothreitol and 2% SDS. Solubilized proteins were immediately loaded on slab gels of 7% acrylamide prepared according to Fairbanks and Avruch [19]. Electrophoresis was carried out with 15 mA for 4–5 h. Gel strips were cut into 54 slices, mixed with an instagel (Packard) and counted 24 h later. Molecular weights of proteins on the gels were calibrated as described by Weber and Osborn [20]. In some experiments, ^{32}P-labeled intermediates of bovine intestinal alkaline phosphatase (64 000 daltons: Boehringer, Mannheim) and of rat heart sarcolemmal membranes were used as markers. Sarcolemmal membranes were isolated as reported by Lamers and Stinis [21] and in these membranes Ca^{2+}-ATPase was the major ^{32}P-labeled protein after incubation at 0°C in the presence of 1 μM $CaCl_2$ and [γ-^{32}P]ATP (De Jonge, H.R., unpublished data). In some experiments, precipitated membrane proteins were separated at alkaline pH according to Laemmli [22] on 7.5% acrylamide gels with a 3% acrylamide stacking gel. For methods of gel staining, drying and autoradiography we refer to Ueda et al. [23]. Hydroxylamine treatment of ^{32}P-labeled membranes was performed as described recently by Harms and Wright [24].

[γ-^{32}P]ATP (2 000 Ci/mmol) and carrier-free ^{32}P-labeled orthophosphate was obtained from the Radiochemical Centre, Amersham. Chlorpromazine and trifluoperazine were gifts from Smith, Kline and French laboratories. All other reagents were analytical grade.

Results

In analogy with other tissues [5–10], a phosphointermediate of Ca^{2+}-ATPase in duodenal plasma membranes should display an acylphosphate character i.e. high sensitivity to alkaline pH and to hydroxylamine. In contrast, hydroxylamine insensitive phosphoester bonds, predominantly phosphoserine, have been reported for active site labeling of alkaline phosphatase and protein kinase catalyzed phosphorylations [25,26]. Therefore, visualization of ATPase phospho-intermediates on acrylamide gels is critically dependent on a low running pH during electrophoresis as in the Fairbanks procedure [19] whereas the other phosphoproteins can be detected at alkaline pH on Laemmli gels [22].

Basolateral membranes

The pattern of ^{32}P incorporation into proteins from duodenal basal lateral membranes is shown in Fig. 1. The conditions chosen ensure optimal steady-state levels of phosphorylation of the phosphoproteins. Apart from radioactivity at the gel front, three distinct peaks are detected migrating with molecular weights of approximately 64 000 (I), 84 000 (II) and 115 000 (III), respectively. The following observations led us to identify proteins bands I and II as phospho-intermediates of alkaline phosphatase: Firstly, protein I comigrated with purified alkaline phosphatase from calf intestine (Boehringer); Secondly, bands I and II disappeared almost completely upon addition of 5 mM β-glycerophosphate to the phosphorylation medium (Fig. 1B and D). In view of the broad range of substrates for alkaline phosphatase, competitive inhibition of ATP hydrolysis can be anticipated by an excess of phosphate esters; Thirdly, proteins I and II can be labeled to a similar extent with inorganic ^{32}P instead of [γ-^{32}P]ATP (results not shown). Ligation of inorganic phosphate to the catalytically active serine group in alkaline phosphatase is a well known phenomenon [25,27]. Finally, the intensity of both ^{32}-labeled protein bands is not affected by hydroxylamine treatment (Table I) nor by alkaline pH on Laemmli gels (see Fig. 3A, line 2).

Phosphoprotein band III, appearing as a shoulder on peak II in Figs. 1A and 1C, displayed the general characteristics of a Ca^{2+}-ATPase phospho-intermediate. This was concluded from the following observations: Firstly, ^{32}P incorporation from [γ-^{32}P]ATP was strongly Ca^{2+}-dependent, both in the presence (Figs. 1A and 1B) and absence of Mg^{2+} (Figs. 1C and 1D) and optimal stimulation was obtained at 1 μM Ca^{2+} (see Table I). Secondly, the phosphoprotein band was sensitive to hydroxylamine (Table I) and disappeared completely at alkaline pH on Laemmli gels (not shown). Thirdly, labeling of band III is only slightly inhibited in the presence of 5 mM β-glycerophosphate (Figs. 1B and 1D), indicating a specific

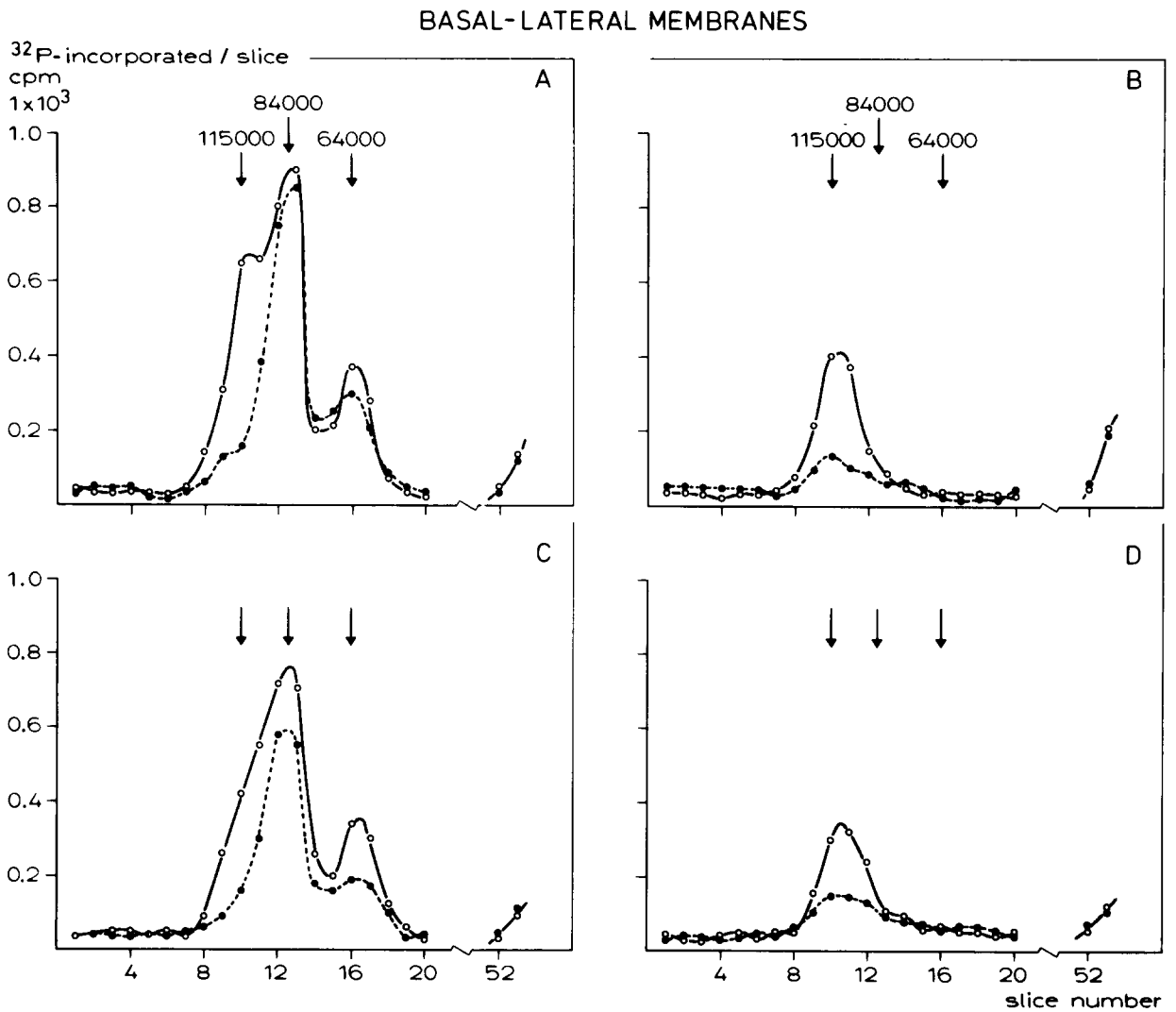

Fig. 1. ^{32}P-labeling pattern of phosphoproteins in crude basolateral membranes of rat duodenal epithelium. Membrane proteins (75 μg) labeled for 30 s at 0°C in the presence of 20 μM [γ-^{32}P]ATP (11 000 dpm/pmol) were separated on acrylamide gels [19] followed by gel slicing and counting as described in Methods. ●——●, Labeling in the absence of Ca^{2+}; ○——○, labeling in the presence of 1 μM Ca^{2+}. A and B: labeling in the presence of Mg^{2+}; C and D: labeling in the absence of 1 mM Mg^{2+}. A and C: no β-glycerophosphate added; B and D: 5 mM β-glycerophosphate added. The reproducibility of this pattern was shown in four separate experiments. The molecular weights of bands I–III were calibrated as described in Methods using calf intestinal alkaline phosphatase (M_r 64 000), phosphorylase *a* (M_r 94 000), endogenous ^{32}P-labeled ($Na^+ + K^+$)-ATPase (M_r 100 000) and the heavy chain of myosin (M_r 200 000) as standards. ^{32}P-labeled Ca^{2+}-ATPase in sarcolemmal membranes of rat heart comigrated exactly with band III.

ATP site. Fourthly, protein III cannot be phosphorylated with inorganic ^{32}P. Finally, band III comigrated exactly with ^{32}P-labeled Ca^{2+}-ATPase present in rat heart sarcolemma (not shown) and ^{32}P incorporation into band III was specifically inhibited by phenothiazines (see also Table I). In an earlier study it was reported that phenothiazines inhibited Ca^{2+}-ATPase activity in basal lateral membranes of rat duodenum [2]. Comparison of Figs. 1B and 1D clearly indicates that 1 μM Ca^{2+} in the presence of 1 mM Mg^{2+} gives a

30 to 40% higher activity in band III than 1 μM Ca^{2+} alone. Such an acceleration of phosphoprotein formation by Mg^{2+} has also been reported for Ca^{2+}-ATPase of red blood cells [7–9] and may be due to an increased transition rate of E_1P into E_2P (see Introduction).

Table I summarizes quantitatively the effects of inhibitors and stimulation of ^{32}P-incorporation into the three bands observed with basolateral membranes. The stimulation of phospho-intermediate formation of band I and II by Zn^{2+} provides additional evidence for the conclusion that band I and II are monomeric units of alkaline phosphatase. This enzyme is a metallo-enzyme requiring Zn^{2+} and Mg^{2+} for maximal activity [28]. From Figs 1A and 1C it can be concluded that ^{32}P incorporation into band I and II can be stimulated either by Mg^{2+} alone or by Ca^{2+} alone. Surprisingly enough, higher levels of free Ca^{2+} (0.2 mM) lead to inhibition of enzyme-labeling which suggests stimulation of phospho-ester hydrolysis (Table I).

Since crude basolateral membranes of rat duodenum are heavily contaminated with fragments of smooth endoplasmic reticulum [13,29] it is of interest to find out whether Ca^{2+}-ATPase is distributed among both membrane populations or exclusively located in one membrane. Therefore, phosphorylation studies were done with basolateral membranes purified further by zonal electrophoresis on density gradients [13]. The purified basolateral membranes used for phosphorylation studies were enriched in ($Na^+ + K^+$)-ATPase activity by a factor of 1.6 (specific activity increased from 21.6 ± 1.7 to 33.9 μmol $P_i \cdot h^{-1} \cdot mg^{-1}$ (n = 7)) and were purified with respect to NADPH-cytochrome c-reductase 13-fold when compared with crude basolateral membranes. These membranes also showed a 1.5-fold increase in Ca^{2+}-ATPase activity and a comparable increase in ^{32}P incorporation into band III (Table II). The ^{32}P-labeling pattern of the purified membranes was identical to Fig. 1A and is therefore not shown. The concomittant increase in ($Na^+ + K^+$)-ATPase, Ca^{2+}-ATPase and phospho-intermediate formation upon removal of smooth endoplasmic reticulum fragments strongly suggests that Ca^{2+}-ATPase is exclusively located in basolateral plasma membranes of duodenal epithelium.

Much attention has been given recently to the role

TABLE I

PHOSPHO-INTERMEDIATE FORMATION OF PROTEIN BANDS I, II AND III IN BASOLATERAL MEMBRANES OF RAT DUODENAL EPITHELIUM

Number of observations in parenthesis.

Phosphorylation conditions	^{32}P incoporation from [γ-^{32}P]ATP (% of control)		
	band I 64 000	II 84 000	III 115 000
1 mM Mg^{2+}	100 [a]	100 [a]	24 ± 5 (4)
1 mM Mg^{2+}, 1 μM Ca^{2+}, 5 mM β-GP [b]	13 ± 7 (4)	11 ± 6 (4)	100 [a]
1 mM Mg^{2+}, 1 μM Ca^{2+}	122 ± 7 (3)	108 ± 4 (3)	119 ± 12 (4)
1 mM Mg^{2+}, 0.2 mM Ca^{2+}	63 ± 13 (5)	73 ± 12 (5)	128 ± 13 (5)
1 mM Mg^{2+}, 10 μM Zn^{2+}	145 ± 12 (3)	152 ± 10 (3)	22 ± 8 (3)
1 mM Mg^{2+}, 0.2 mM Zn^{2+}	264 ± 22 (3)	294 ± 27 (3)	30 ± 6 (3)
Sodium acetate treatment [c]	84 ± 5 (4)	81 ± 5 (4)	80 ± 6 (4)
Hydroxylamine treatment [c]	86 ± 4 (4)	79 ± 5 (4)	13 ± 4 (4)
0.1 mM chlorpromazine [d]	97 ± 7 (3)	92 ± 8 (3)	17 ± 5 (3)
0.1 mM trifluoperazine [d]	106 (2)	99 (2)	24 (2)

[a] Arbitrarily chosen as control value (100%).

[b] β GP, β-glycerophosphate.

[c] Membranes were first phosphorylated in the presence of 1 mM Mg^{2+} plus 1 μM Ca^{2+} and then treated with sodium acetate or hydroxylamine in sodium acetate.

[d] Phosphorylation in the presence of 1 mM Mg^{2+}, 1 μM Ca^{2+} and 0.1 mM drug.

TABLE II

STEADY-STATE LEVELS OF PHOSPHATE INCORPORATION INTO Ca^{2+}-ATPase OF CRUDE BASOLATERAL AND OF PURIFIED BASOLATERAL MEMBRANES

Comparison with membranes of the erythrocyte.

	Ca^{2+}-ATPase		
	Specific activity [a] (μmol P_i/h per mg protein)	^{32}P incorporated [b] (pmol/mg protein	Turnover number [c] (ATP/min)
Crude basolaterals	1.4 ± 0.3 (7)	1.1 ± 0.2 (4)	21 000
Purified basolaterals	2.1 ± 0.4 (7)	1.5 ± 0.2 (3)	23 000
Erythrocyte	1.5–2.0 (ref. 5)	1.2 (Ref. 9)	21 000–28 000

[a] ATP hydrolysis stimulated by 1 μM Ca^{2+} as described in Methods.

[b] Following separation of ^{32}P-labelled membrane proteins, the peak area of Ca^{2+}-ATPase, band III, appearing upon stimulation with 1 μM Ca^{2+} in the presence of 5 mM β-glycerophosphate (Fig. 1B), is measured and gives the total amount of phosphate incorporated.

[c] This calculation assumes, that under optimal steady-state labeling conditions one mol of phosphate is bound per mol enzyme.

of calmodulin in activating Ca^{2+}-ATPase of erythrocytes [5,30,31]. Addition of 10 μg calmodulin (bovine brain) to 200 μg crude basolateral membrane protein stimulated the activity of Ca^{2+}-ATPase by 58 ± 16% (n = 5), whereas the background Mg^{2+}-ATPase activity did not change. We were unable to detect significant changes in the level of ^{32}P-incorporation into band III by exogenous calmodulin. This result is not unexpected since it has been reported recently that in erythrocytes calmodulin stimulates

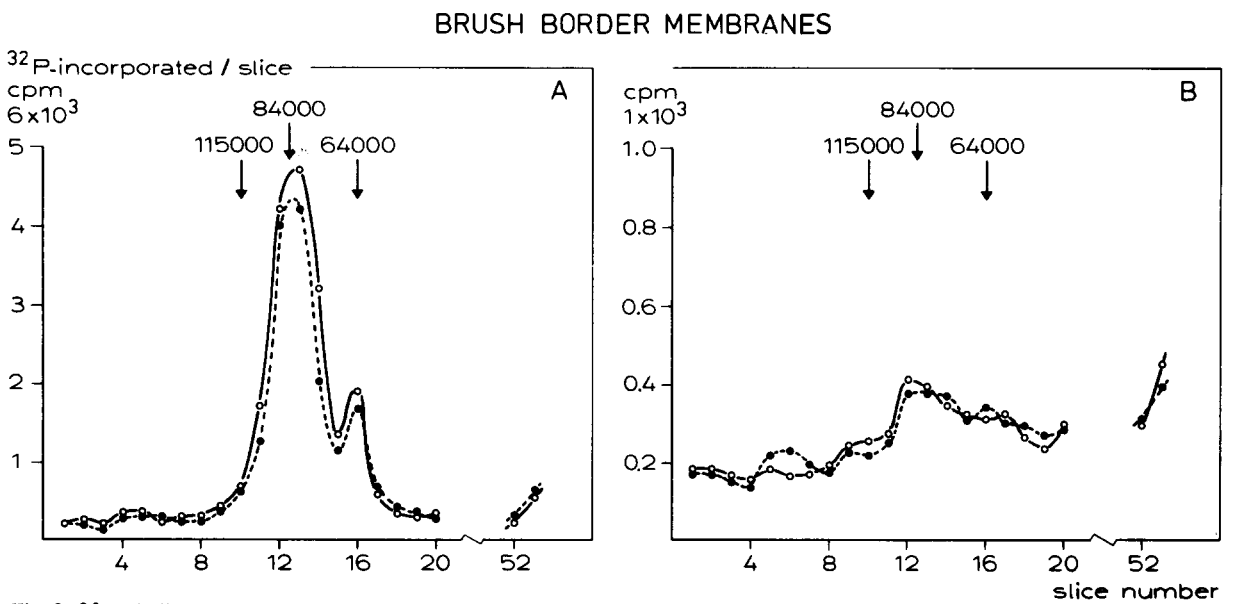

Fig. 2. ^{32}P-labeling pattern of phosphoproteins in brush border membranes of rat duodenal epithelium. Membrane proteins (60 μg) labeled for 30 s at 0°C in the presence of 1 mM $MgCl_2$ and 20 μM [γ-^{32}P]ATP (10 000 dpm/pmol) were separated on acrylamide gels [19] followed by gel slicing and counting. •———•, Labeling in the absence of Ca^{2+}; o———o, labeling in the presence of 1 μM Ca^{2+}. A: no β-glycerophosphate added; B: 5 mM β-glycerophosphate added. For further comments, see Fig. 1. Note the difference in scale at the ordinate of Figs. 2A and 2B.

Ca^{2+}-ATPase up to 280% while the effect on phospho-enzyme formation was only 30% [30].

Brush border membranes

The results of phosphorylation experiments with purified leaky brush border membranes are given in Fig. 2 and Fig. 3. Fig. 2 shows the pattern of ^{32}P-labeling of brush border protein obtained in a way similar to the pattern in Fig. 1. Two major peaks of radio-activity were found at positions identical to bands I and II in Fig. 1. The same bands could also be visualized on autoradiographs of Laemmli gels (Fig. 3), confirming the stability of these phosphoproteins at alkaline pH. The total amount of ^{32}P incorporated into the two bands expressed per mg of membrane protein was on the average 7-fold higher in brush borders than in basolaterals (see Fig. 3A, lane 1 and 2). This is in agreement with a distribution ratio of about 10 for alkaline phosphatase distribution among brush border and basolateral membranes [4].

In general, the response of the steady state labeling levels of band I and II in both membranes to activators and inhibitors was qualitatively similar. In brush borders, the formation of phospho-enzyme was also inhibited by excess β-glycerophosphate (Fig. 2B,

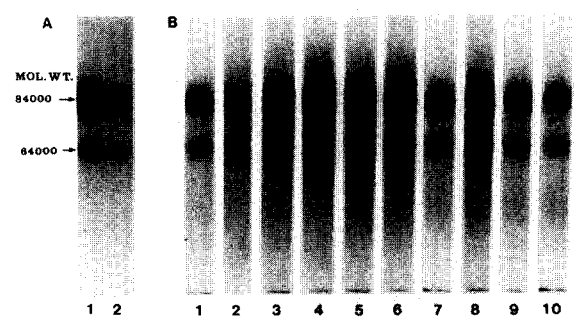

Fig. 3. Autoradiograph of ^{32}P-labeled brush border and basolateral membrane proteins separated on an acrylamide-SDS slab gel according to Laemmli [22]. Membranes were labeled with [γ-^{32}P]ATP as described in the legend of Fig. 2. A: Comparison between brush border membranes (lane 1) and basolateral membranes (lane 2) phosphorylated in the presence of 200 μM Zn^{2+}. B: Brush border membranes. Lane 1: no addition; lane 2: 5 mM β-glycerophosphate; lane 3: 10 μM Zn^{2+}; lane 4: 200 μM Zn^{2+}; lane 5: 5 mM theophylline; lane 6: 5 mM theophylline, 10 μM Zn^{2+}; lane 7: 1 μM Ca^{2+}; lane 8: 200 μM Ca^{2+}; lane 9: 0.1 mM chlorpromazin; lane 10: 50 μg calmodulin + 1 μM Ca^{2+}.

Fig. 3B, lane 2), whereas Zn^{2+} (Fig. 3B, lane 3 and 4), theophylline (Fig. 3B, lane 5) and especially Zn^{2+} plus theophylline (Fig. 3B, lane 6) stimulated ^{32}P incorporation. Since theophylline is a potent inhibitor of alkaline phosphatase [32] the last observation means that theophylline inhibits phospho-intermediate hydrolysis rather than formation. ^{32}P incorporation into brush border protein was slightly stimulated by 1 μM Ca^{2+} (8–13%, n = 3, Fig. 3B, lane 7) and more strongly by 0.2 mM Ca^{2+} (30%, n = 3, Fig. 3B, lane 8). This stimulation at high Ca^{2+} levels is not seen with basolateral membranes (Table I). The reason for this difference is unclear in view of the homology between alkaline phosphatase activities in both membranes [2,4]. Fig. 3B also demonstrates the insensitivity of alkaline phosphatase to phenothiazines and calmodulin (lane 9 and 10), which indicates that the Ca^{2+}-sensitivity of this enzyme is not calmodulin mediated.

Despite the fact that we tried various labeling conditions, we were unable to show a specific Ca^{2+}-ATPase intermediate in brush border membranes. Although it is tempting to conclude that all Ca^{2+} stimulated ^{32}P incorporation is into alkaline phosphatase, it may well be that a small amount of ^{32}P incorporation into a 115 000-dalton protein goes undetected because of the high level of alkaline phosphatase intermediates, whose activity is not completely abolished with β-glycerophosphate (Fig. 2B and Fig. 3B, lane 2).

Discussion

In this study we have demonstrated on acrylamide gels phospho-intermediates of Ca^{2+}-ATPase and alkaline phosphatase in basolateral plasmamembranes of duodenal cells. The results clearly indicate that both enzymes are separate entities, and this is fully in support of an earlier study in which the same conclusion was reached on the basis of substrate specificities and the effects of inhibitors on the two enzymes [2].

The approach followed enabled us to characterize some chemical properties of the phospho-intermediates which formed the basis for the distinction between the two enzymes. Whereas phosphoenzyme formation of Ca^{2+}-ATPase in membranes of red blood cells [5] or muscle [6] is usually quantified by millipore filtration, a similar approach was unsuccessful

with duodenal membranes due to the high background of ^{32}P label incorporated into alkaline phosphatase. Although no thorough kinetic analysis of the phosphoenzyme formation has been done, the results from this study are in agreement with ^{32}P labeling studies of Ca^{2+}-ATPase in other tissues [5,6]. The phosphoenzyme formation of intestinal Ca^{2+}-ATPase was almost completely Ca^{2+}-dependent even in the presence of 1 mM Mg^{2+} and its Ca^{2+}-sensitivity was already optimal at 1 μM free Ca^{2+}. Upon removal of contaminating endoplasmic reticulum fragments by preparative electrophoresis, Ca^{2+}-ATPase activity and the ^{32}P-labeled intermediate copurified with ($Na^+ + K^+$)-ATPase which indicates that Ca^{2+}-ATPase is only present in basolateral plasma membranes.

An important observation in this study was that Ca^{2+}-ATPase of duodenal basolaterals, comigrated on SDS gels with the Ca^{2+}-ATPase activity of rat heart sarcolemma. This suggests that the apparent molecular weights are identical. In our hands, the apparent molecular weight was about 115 000–120 000. Very recently, Ca^{2+}-ATPase from membranes of erythrocytes and from heart sarcolemma have been purified by calmodulin affinity chromatography by Niggli et al. [33,34]. The molecular weights of the purified Ca^{2+}-ATPases were 138 000 [34]. In view of the similar electrophoretic mobility of Ca^{2+}-ATPase from duodenal membranes and heart sarcolemma, it is likely that our molecular weight value is an underestimate due to the poor resolution obtained in gels with acidic pH.

Remarkably enough no other hydroxylamine sensitive phospho-intermediates of an ATPase reaction, besides Ca^{2+}-ATPase and ($Na^+ + K^+$)-ATPase, could be detected in duodenal membranes in spite of the high background of Mg^{2+}-ATPase activity measured in ATPase assays [2]. Although the Mg^{2+}-ATPase background may be a sum of a variety of ATP hydrolyzing enzymes, none of these ATPases seemed capable of phospho-intermediate formation in sufficient amounts to be detected. This property of Mg^{2+}-ATPase activity is shared with the F_1-ATPase from mitochondria [35]. Moreover, the contribution of more slower reactions to the ^{32}P-labeling pattern, such as protein kinase catalyzed phosphorylation, is also negligible [36].

A strong inhibition of phospho-intermediate formation of Ca^{2+}-ATPase was observed with 0.1 mM chlorpromazine or trifluoperazine. In view of recent observations by Hinds et al. [37] these concentrations may have been too high to explain their effects solely on the basis of selective antagonism of calmodulin-induced stimulation of Ca^{2+}-ATPase. With concentrations of 0.1 mM and up, phenothiazines may induce disruption of the membrane lipid environment and thereby inhibit calmodulin-independent basal Ca^{2+}-ATPase activities. This possibility is supported by our observation that 0.1 mM chlorpromazine also inhibits 30% of background Mg^{2+}-ATPase activity and 60% of ($Na^+ + K^+$)-ATPase activity in our ATPase assays (unpublished data). Similar effects of chlorpromazine have been reported on Mg^{2+}-ATPase and ($Na^+ + K^+$)-ATPase in rat liver membranes [38]. Although phenothiazines can be used as selective antagonists of calmodulin-stimulated Ca^{2+}-ATPase activity, we did not attempt a dose-response study since calmodulin had a relatively small effect on Ca^{2+}-ATPase activity in duodenal membranes. The minor effect of calmodulin in our preparation may be a consequence of the high endogeneous level of this protein in intestinal basolateral membranes (about 10 to 20 μg per mg of membrane protein; unpublished data).

Finally, considering the possible importance of Ca^{2+}-ATPase in the active transport of Ca^{2+} across the duodenum, it is interesting to calculate the turnover number from the phosphorylation data and the specific activity. This has been done in Table II. We could also calculate similar turnover numbers for Ca^{2+}-ATPase from erythrocyte membranes from phosphorylation studies by Rega and Garrahan [9] (Table II). In control studies we have estimated the amount of ^{32}P incorporated into ($Na^+ + K^+$)-ATPase of duodenal basolateral membranes and found a labeling efficiency similar to that reported recently by Harms and wright [24]. These authors calculated from phosphorylation of ($Na^+ + K^+$)-ATPase, that there are about $1.5 \cdot 10^5$ Na^+ pumps per intestinal cell. Since the amount of Ca^{2+}-ATPase and its maximum phosphorylation is about 15-times less than the values for ($Na^+ + K^+$)-ATPase, this would mean that there are about 10^4 Ca^{2+}-pump sites per duodenal cell. Using this number of pump sites per cell, the turnover number ($20 \cdot 10^3$ mol ATP per min) from Table II and assuming that one calcium ion is transported per cycle of the pump [33,34], then we calculate that

$2 \cdot 10^8$ calcium ions can be transported out of each duodenal cell per min. Since there are about 10^8 epithelial cells per cm^2 small intestine [24], the maximal rate of Ca^{2+}-transport across the duodenum based on the Ca^{2+}-ATPase activity amounts to about 2 μ-equiv. $\cdot h^{-1} \cdot cm^{-2}$. The observed rate of Ca^{2+}-transport across short-circuited duodenum is 0.1 μ-equiv. $\cdot h^{-1} \cdot cm^{-2}$ [39]. These calculations suggest that the Ca^{2+}-ATPase activity in rat duodenum is more than adequate to account for active Ca^{2+} absorption.

In plasma membranes of rat duodenal epithelium phospho-intermediate forms of alkaline phosphatase could be demonstrated easily. The native form of rat intestinal alkaline phosphatase has been described as a dimer of nearly identical subunits existing in different subforms running in a molecular weight range between 124 000 and 150 000 on SDS-acrylamide gels [4,40]. Following treatment with reducing agents the enzyme is separated into monomers [4] with slightly differing molecular weights [40]. In our study usually 60 to 75% (n = 8) of alkaline phosphatase covalently labeled in the presence of [γ-^{32}P]ATP was found at a 84 000-dalton position on Laemmli gels whereas the residual part comigrated with a commercial preparation of bovine intestine with a molecular weight of 64 000. In a few preparations, however, this ratio changed in favour of the lower molecular weight form. The width of both alkaline phosphatase bands most probably results from a microheterogeneity of the enzyme related to its high carbohydrate content [40,41].

All attempts to discover a phospho-intermediate form of Ca^{2+}-ATPase in purified brush borders failed. This observation is in support of an earlier reached conclusion that Ca^{2+}-ATPase is exclusively located on the basolateral side of duodenal cells [2].

The demonstration of a Ca^{2+}ATPase with properties of a Ca^{2+} pump has several implications: Firstly, it demonstrates that this enzyme possesses a more universal distribution and that it is probably involved in Ca^{2+} transport across epithelia or in maintaining low intracellular levels of free Ca^{2+}; secondly, it justifies a further study of ATP dependent Ca^{2+} transport in inside-out vesicles of duodenal basolateral membranes as recently reported by Hildman et al. [42] and by Ghijsen and Van Os [43]; thirdly, the role of this enzyme in vitamin D-3 dependent Ca^{2+} absorption has to be studied, and it is of particular interest whether vitamin D-3 is able to regulate the activity of duodenal Ca^{2+}-ATPase.

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