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PHOSPHORYLATED INTERMEDIATES OF (Ca^{2+} + Mg^{2+})-ATPase AND ALKALINE PHOSPHATASE IN RENAL PLASMA MEMBRANES

HUMBERT DE SMEDT, JAN B. PARYS, ROGER BORGHGRAEF and FRANK WUYTACK

Laboratorium voor Fysiologie, Campus Gasthuisberg, Katholieke Universiteit Leuven, B-3000 Leuven (Belgium)

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Renal basal-lateral and brush border membrane preparations were phosphorylated in the presence of [γ-^{32}P]ATP. The ^{32}P-labeled membrane proteins were analysed on SDS-polyacrylamide gels. The phosphorylated intermediates formed in different conditions are compared with the intermediates formed in well defined membrane preparations such as erythrocyte plasma membranes and sarcoplasmic reticulum from skeletal muscle, and with the intermediates of purified renal enzymes such as (Na^+ + K^+)-ATPase and alkaline phosphatase. Two Ca^{2+}-induced, hydroxylamine-sensitive phosphoproteins are formed in the basal-lateral membrane preparations. They migrate with a molecular radius M_r of about 130 000 and 100 000. The phosphorylation of the 130 kDa protein was stimulated by La^{3+}-ions (20 μM) in a similar way as the (Ca^{2+} + Mg^{2+})-ATPase from erythrocytes. The 130 kDa phosphoprotein also comigrated with the erythrocyte (Ca^{2+} + Mg^{2+})-ATPase. In addition in the same preparation, another hydroxylamine-sensitive 100 kDa phosphoprotein was formed in the presence of Na^+. This phosphoprotein comigrates with a preparation of renal (Na^+ + K^+)-ATPase. In brush border membrane preparations the Ca^{2+}-induced and the Na^+-induced phosphorylation bands are absent. This is consistent with the basal-lateral localization of the renal Ca^{2+}-pump and Na^+-pump. The predominant phosphoprotein in brush border membrane preparations is a 85 kDa protein that could be identified as the phosphorylated intermediate of renal alkaline phosphatase. This phosphoprotein is also present in basal-lateral membrane preparations, but it can be accounted for by contamination of those membranes with brush border membranes.

Introduction

In a previous study [1] we have demonstrated a high-affinity (Ca^{2+} + Mg^{2+})-ATPase in detergent extracts of renal plasma membranes. This ATPase has a specific requirement for Ca^{2+} in the micromolar range and can be considered as the enzymatic expression of the renal calcium pump. The distribution of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase along the rabbit nephron was recently described [2]. The subcellular localization was defined in highly purified basal-lateral membrane fractions [3].

Basal-lateral membrane vesicles accumulate Ca^{2+} in an ATP-dependent way [4]. The concomitant (Ca^{2+} + Mg^{2+})-ATPase activity in the same preparations, however, amounts to only a few percent of the total ATPase activity in the preparation.

In order to obtain specific molecular data for the renal (Ca^{2+} + Mg^{2+})-ATPase which represents only a minor fraction of the proteins in the membrane preparations, we have studied the phos-

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

phoprotein intermediates which are formed in the presence of [γ-^{32}P]ATP. The phosphorylated intermediates are analysed by SDS-polyacrylamide gel electrophoresis. We report in this study data about the phosphoproteins found in renal plasma membranes, the ionic requirements for their formation, their biochemical characteristics and subcellular distribution.

The data for kidney preparations are compared with the phosphorylated intermediates observed in inside-out vesicles of red blood cell membranes, sarcoplasmic reticulum of skeletal muscle and in purified renal enzyme preparations such as alkaline phosphatase and ($Na^+ + K^+$)-ATPase.

Materials and Methods

Membrane preparation and enzyme purification

Hog kidneys, obtained from a local slaughterhouse, were perfused with ice-cold isotone salt solution. The kidney cortex was dissected and stored at −70°C until needed. The membrane preparations were assayed as previously described for enzymes shown to be characteristic for brush-border microvilli: alkaline phosphatase (EC 3.1.3.1) (determined using an optimized kinetic test; Merckotest 3344 Merck, A.G.), aminopeptidase M (EC 3.4.11.2) [5]; basal-lateral membranes: ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) [6]; mitochondria: cytochrome *c* oxidase (EC 1.9.3.1) [7]; and endoplasmic reticulum: rotenone-insensitive NADH-cytochrome *c* reductase (EC 1.6.99.3) [8]. Protein was determined following the method of Lowry et al. [9] after precipitation of the membrane protein by 10% (w/v) ice-cold trichloroacetic acid.

Brush border membrane vesicles were prepared as described elsewhere [10]. The preparation was enriched 10-fold in brush border markers versus the original homogenate. The enrichment of ($Na^+ + K^+$)-ATPase in this preparation was 0.5–0.6-fold.

A crude basal-lateral membrane preparation was obtained as described earlier [1]. This preparation is enriched 4–6-fold in ($Na^+ + K^+$)-ATPase and 2–3fold in the brush border marker enzymes. The crude basolateral membranes were further purified by a centrifugation step on a discontinuous density gradient. The gradient consists of three successive layers buffered with 10 mM triethanolamine-HCl (pH 7.5): from the bottom to the top (a) a cushion of 6 ml of sucrose 60% w/v; (b) 20 ml of sucrose 32% w/v + Ficoll 400, (Pharmacia Fine Chemicals) 3% w/v and (c) 10 ml of sucrose 20% w/v + Ficoll 400, 5% w/v.

Each gradient was loaded with 2 ml of a sample (protein concentration: 5 mg/ml) and centrifuged in a swinging bucket rotor (Beckman SW 28) for 3 h at 27000 rpm (130000 × *g*). This method is an adaptation of a method described for the preparation of sinusoidal plasma membranes from rat liver *. The highest enrichment in basal-lateral membrane markers was found in the fraction on the second interphase between the 20% sucrose + 5% Ficoll and the 32% sucrose + 3% Ficoll layers. The vesicles were washed and finally suspended in a medium containing 100 mM KCl, 30 mM imidazole-HCl (pH 6.8), 100 mM sucrose and 5 mM Tris-azide. The enrichment factors versus the homogenate for this preparation were: 9.1 ± 0.5 (6) for ($Na^+ + K^+$)-ATPase, 1.8 ± 0.2 (6) for aminopeptidase M; 2.3 ± 0.2 (6) for alkaline phosphatase; 1 ± 0.1 (6) for NADH-cytochrome *c* reductase and 0.3 ± 0.3 (3) for cytochrome *c* oxidase. The values are means ± S.E.; the number of observations is given in parentheses.

In some experiments we have used Triton X-100 extracts of the plasma membrane preparations. The membranes were incubated with the detergent at 0°C for 10 min. The conditions were: protein concentration: 4 mg/ml; protein/detergent ratio = 1/1; KCl, 100 mM; imidazole-HCl, 30 mM (pH 6.8). After incubation with the detergent, the preparation was centrifuged at 50000 rpm for 30 min in a Beckman Ti 75 rotor. The supernatant was used in phosphorylation experiments. It contained about 30% of the protein. By this procedure, the ($Ca^{2+} + Mg^{2+}$)-ATPase is solubilized to a larger extent than other proteins, e.g. Mg^{2+}-ATPase. In Triton-extracts of basal-lateral membranes, ($Ca^{2+} + Mg^{2+}$)-ATPase activity amounts to 40 nmol $P_i \cdot (mg\ protein)^{-1} \cdot min^{-1}$ at 37°C. Moreover this activity could be stimulated 2-fold by calmodulin. In Triton X-100 extracts of brush border membranes there was no indication for a high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase.

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Alkaline phosphatase was purified from porcine kidney by *n*-butanol extraction, aceton fractionation and gel chromatography on Sephacryl S-200, by the procedure of Mössner et al. [11]. The preparation obtained after gel chromatography was enriched 1700-fold in alkaline phosphatase versus the homogenate. Further purification steps were omitted. In some experiments a commercial preparation of alkaline phosphatase from intestine (Boehringer) was used.

$(Na^+ + K^+)$-ATPase was purified from porcine kidney outer medulla as described earlier [6].

Fragmented sarcoplasmic reticulum from skeletal muscle was prepared from white skeletal muscles from the leg and back of the rabbit [12].

Inside-out vesicles of erythrocytes were prepared from porcine blood as described by Steck and Kant [13] but the final Dextran gradient step was omitted.

All preparations were kept at −70°C until used.

Phosphorylation experiments

The phosphorylation reactions, SDS-polyacrylamide slab gel electrophoresis at pH 2.4, treatment with hydroxylamine and autoradiography was done exactly as described earlier [14]. The phosphorylation reactions were performed in Eppendorf 3810 tubes on ice, for time periods as indicated in the legends of Table I and the figures. The reaction was started by adding 6 μM [γ-^{32}P]ATP at 13 Ci/mmol to a medium containing: 100 mM KCl, 30 mM imidazole-HCl (pH 6.8) and either 50 μM $CaCl_2$ or 0.5 mM EGTA (potassium salt) in a total volume of 200 μl. Other components present in the different conditions, are indicated in the legends to the figures. The total amount of membrane-protein present was 0.2–0.5 mg for renal plasma membranes and for erythrocyte membranes; 0.4 μg for sarcoplasmic reticulum and 0.3 μg for renal alkaline phosphatase and $(Na^+ + K^+)$-ATPase. The reaction was stopped by 0.5 ml of a stop solution containing: 10% trichloroacetic acid, 50 mM phosphoric acid and 0.5 mM ATP.

Boehringer Combithek calibration standards were used for estimation of M_r. The part of the slab gels with the M_r reference proteins, was cut off after electrophoresis and stained separately with Coomassie brilliant blue. M_r values were obtained from curves relating M_r values of standard proteins to their corresponding R_F values.

Densitometric scans of the autoradiography plates were made by means of the LKB 2202 Ultra Scan Laser densitometer.

Transport studies

Uptake of $^{45}Ca^{2+}$ was assayed by a Millipore filtration technique as described by Kinne et al. [15]. The basic components of the incubation medium were 100 mM KCl, 100 mM sucrose, 30 mM imidazole-HCl (pH 6.8), 1 mM EGTA, 5 mM azide (Tris salt). The concentration of free Mg^{2+} (1 mM) and free calcium (10 μM) were calculated as in Ref. 16. $^{45}Ca^{2+}$ was 30 μCi/ml. ATP (di-Tris salt) and precipitating anions were added as indicated in the legend to Fig. 1. The stop solution contained 150 mM KCl, 100 mM sucrose, 30 mM imidazole-HCl (pH 6.8), 1 mM EGTA and 5 mM Mg^{2+}. Millipore filters (HAWP 02500) with 0.45 μm pore size were used. In the experiment 20 μl of membrane suspension (2.2 mg/ml) was added to 600 μl of the incubation medium, kept at 37°C. At different time intervals 100 μl samples were removed from the incubation suspension and diluted into 1 ml of ice-cold stop solution and immediately filtered. The filters were washed with 4 ml of ice-cold stop solution. The radioactivity remaining on the filters was counted by standard liquid-scintillation techniques.

[γ-^{32}P]ATP (PB 168) and ^{45}Ca (ES 3) were from the Radiochemical Centre Amersham, U.K. The [γ-^{32}P]ATP was lyophylized and redissolved in water immediately before use.

Results

Basal-lateral membranes

The Ca^{2+}-uptake capacity of the basal-lateral membrane preparation used in this study, is illustrated in Fig. 1. In the absence of ATP, only 2 to 3 nmol calcium per mg protein are taken up in the vesicles. Precipitating anions, 5 mM oxalate or 40 mM phosphate, had no effect on this value (data not shown). In the presence of ATP, the calcium pump is activated and 7-times more Ca^{2+} is accumulated in the vesicles. A maximum is reached after about 10 min. If precipitating anions, oxalate (5 mM) or phosphate (40 mM) are present in the medium, the accumulation of

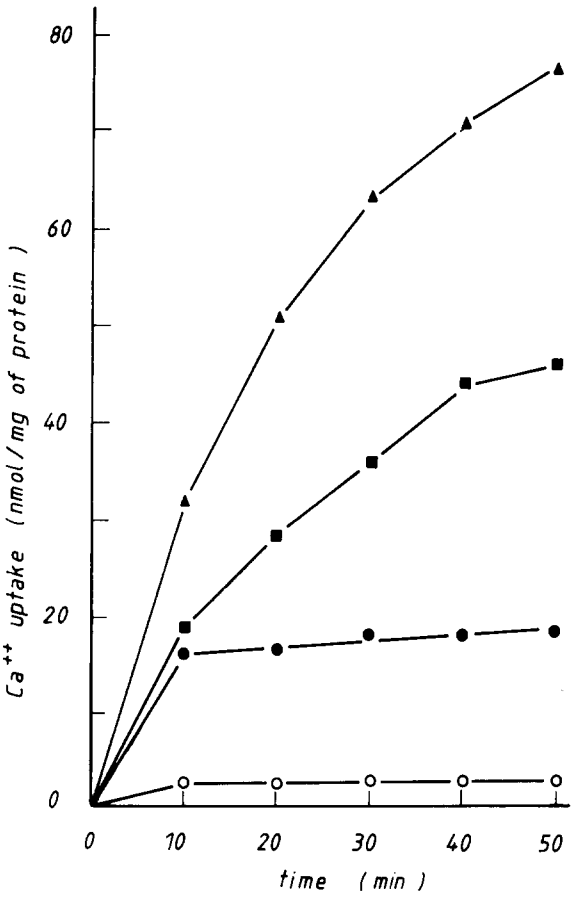

Fig. 1. ATP-driven transport of calcium into basal-lateral plasma membrane vesicles. The membranes were suspended in 100 mM KCl, 100 mM sucrose, 30 mM imidazole-HCl (pH 6.8) and 5 mM azide (Tris salt). The incubation medium contained the same solutes and in addition 1 mM EGTA, and 5 mM ATP (●, ■, ▲) or no ATP (○). Precipitating anions were present at a concentration of 40 mM for phosphate (▲) and 5 mM for oxalate (■). The pH was adjusted to 6.8 with Tris-base. Mg^{2+} and Ca^{2+} were added to a concentration of 1 mM for free Mg^{2+} and 10 μM for free Ca^{2+}. The incubation temperature was 37°C.

calcium is much higher. The free Ca^{2+} concentration in the vesicles is kept low by the formation of a precipitate and further uptake by the calcium pump can go on [17]. In the presence of phosphate, more than 75 nmol of Ca^{2+} per mg of protein were accumulated after 50 min. Since the precipitating anions are added at the outside of the vesicles, the different potency in stimulation of the calcium uptake, is probably due to a difference in the permeability of the vesicles for the anions. In our preparation, 40 mM phosphate is more effective in stimulating the calcium uptake than 5 mM oxalate. This result is in agreement with recent observations made in smooth muscle plasmalemmal membranes, but the reverse was seen in the smooth muscle endoplasmic reticulum [28]. For various cell types, the permeability for oxalate is apparently much higher in the endoplasmic reticulum than in the plasma membrane [28–30]. The oxalate stimulation of the calcium uptake in our preparation is small compared to the oxalate stimulation observed in renal microsomal fractions [29], but it could still indicate a contamination of our preparation with endoplasmic reticulum. On the other hand, it should be mentioned that a 2-fold stimulation of Ca^{2+}-uptake by 5 mM oxalate was also reported in plasma membranes from mammalian erythrocytes [31].

It should be pointed out that only the closed inside-out oriented vesicles contribute to the uptake of calcium. In the presence of a calcium ionophore (e.g. A23187), no ATP-dependent uptake of calcium occurs (data not shown). The transport experiment in Fig. 1 clearly demonstrates the presence of an ATP-driven calcium pump in the basal-lateral plasma membranes.

The enzymatic equivalent for this calcium pump is a high affinity (Ca^{2+} + Mg^{2+})-ATPase. However, we could not detect this activity in our basal-lateral membrane preparation, due to the high background activity of Mg^{2+}-ATPase in this preparation. The (Ca^{2+} + Mg^{2+})-ATPase becomes detectable only after an extraction procedure with Triton X-100 as described in the methods. Even after partial purification by mixed micelle chromatography [1], the (Ca^{2+} + Mg^{2+})-ATPase constitutes only a small fraction of the membrane proteins among which (Na^+ + K^+)-ATPase and Mg^{2+}-ATPase.

The technique of phosphorylation in the presence of [γ-^{32}P]ATP and analysis of the phosphorylated intermediates by SDS-polyacrylamide gel electrophoresis offers the possibility to obtain specific molecular information. The formation of Ca^{2+}-induced phosphorylated proteins is easily detectable. Moreover, in agreement with De Jonge et al. [18], we did not observe the formation of phospho-intermediates corresponding to a Mg^{2+}-ATPase activity. Therefore, there is no need to

extract and purify (Ca^{2+} + Mg^{2+})-ATPase for the phosphorylation studies. The vesicular preparation of basal-lateral membranes can be used directly. In some experiments we have used Triton X-100 extracts to eliminate effects of sidedness that could occur in the vesicle preparations.

An autoradiogram of the phosphorylated proteins of a preparation of basal-lateral membrane vesicles is shown in Fig. 2. The composition of the phosphorylation medium is indicated in the legend of the figure. The preparations in lane 8 (alkaline phosphatase from hog kidney), lane 9 (erythrocyte membrane vesicles) and lane 10 (sarcoplasmic reticulum of skeletal muscle) are used as markers in this experiment. Their M_r values were determinated as described in the methods. In the basal-lateral membrane preparation, two phosphorylations have a specific requirement for calcium. They migrate with a molecular radius of approx. 130000 and 100000, respectively (lane 2). If calcium is complexed by EGTA (lane 1) both bands are absent. The phosphorylation level of the 130 and 100 kDa proteins is not changed significantly by the presence of 1 mM Mg^{2+} (lane 6). They are not phosphorylated in the presence of Mg^{2+} alone without calcium (lane 5). A remarkable effect is observed in the presence of lanthanum ions. The 130 kDa band is strongly stimulated (lane 3: 20 μM La^{3+}; and lane 4; 100 μM La^{3+}).

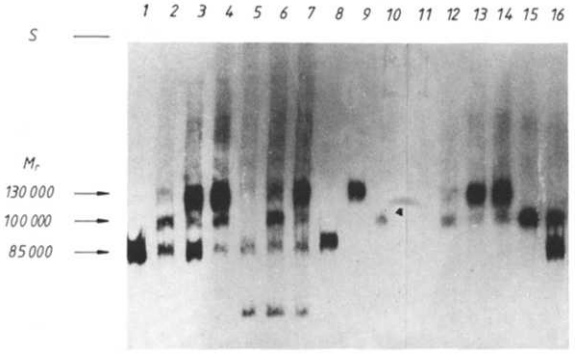

Fig. 2. Autoradiogram of ^{32}P-labeled basal-lateral membrane proteins. The phosphorylation was done for 60 s at 0°C. The reaction was started by addition of [γ-^{32}P] ATP (6 μM) at 13 Ci/mmol. The basic medium contained 30 mM imidazole-HCl (pH 6.8) and 100 mM KCl (lanes 1–14) or 100 mM NaCl (lanes 15,16). In addition, the following solutes were present: EGTA (K^+ salt) (0.5 mM): lanes 1, 5, 11, 15 and 16; Ca^{2+} (50 μM): lanes 2–4, 6–10 and 12–14; Mg^{2+} (1 mM): lanes 5–7; La^{3+} (20 μM): lanes 3, 7 and 13; La^{3+} (100 μM): lanes 4 and 14; β-glycerophosphate (5 mM): lanes 11–15. The total volume including the samples was 200 μl. The samples were 0.3 μg of renal alkaline phosphatase in lane 8, 0.2 mg of erythrocyte plasma membranes in lane 9, 0.4 μg of sarcoplasmic reticulum in lane 10 and 0.5 mg of renal basal-lateral plasma membrane proteins in the other lanes. SDS-polyacrylamide slab gels (5% acrylamide) were run according to Ref. 26 for 3 h.

In the same figure it can be seen that there is a third phosphorylation band, which migrates with the same M_r (approx. 85000) as the renal alkaline phosphatase. This band has no specific requirement for Ca^{2+}. It is reduced in the conditions where divalent or trivalent ions are present in relatively high concentration for example lane 4 (100 μM La^{3+}) or lane 5, 6, 7 (1 mM Mg^{2+}). The highest intensity for this band is observed in the presence of EGTA (lane 1). The 85 kDa band is completely inhibited by the presence of 5 mM β-glycerophosphate in the phosphorylation medium (lanes 11–15). The conditions in lanes 11–14 are identical to the conditions in lanes 1–4 except for the presence of β-glycerophosphate.

In lane 15 and 16, K^+ was replaced by Na^+. An Na^+-dependent phosphorylation appears at approx. 100 kDa. This phosphorylation occurs in the absence of Ca^{2+} (EGTA is present) in contrast to the calcium-requiring 100 kDa band discussed earlier. A preparation of purified (Na^+ + K^+)-ATPase gives a phosphorylated intermediate that comigrates with this 100 kDa band (not shown in the figure).

In the presence of Mg^{2+} (1 mM) several additional bands appear. In the lower M_r range there are two additional phosphoproteins with M_r values of about 55000 and 45000, respectively. The 45 kDa band migrated off the gel in the conditions of the experiment in Fig. 1. These conditions (duration of the electrophoresis was 3 h) were chosen to improve the resolution in the high M_r range. The Mg^{2+}-dependent low M_r phosphoproteins are further discussed in the data illustrated in Table I.

For further characterization of the phosphorylated proteins, the sensitivity towards hydroxylamine was examined. The most important bands are illustrated in Fig. 3. The calcium-dependent phosphoproteins at M_r 130000 and M_r 100000 are completely abolished when the pellets of the phosphorylated proteins are treated with hydroxyl-

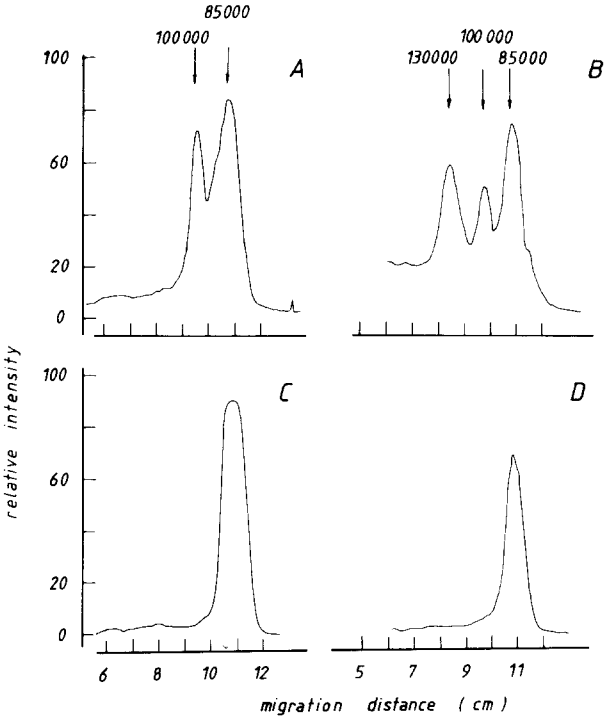

Fig. 3. The effect of hydroxylamine on the phosphorylated proteins in renal basal-lateral plasma membranes. The phosphorylations and analysis on polyacrylamide gel were as described in the legend of Fig. 2. The incubation media contained 30 mM imidazole-HCl (pH 6.8) and in addition 100 mM NaCl and 0.5 mM EGTA in (A) and (C) and 100 mM KCl, 50 μM Ca^{2+} and 100 μM La^{3+} in (B) and (D). The pellets of precipitated phosphoproteins were incubated for 10 min at room temperature in 1 ml of a solution containing 150 mM hydroxylamine hydrochloride and 150 mM sodium acetate at pH 6 (C and D) or in a control solution containing 150 mM Tris-HCl and 150 mM sodium acetate at pH 6 (A and B). The figure represents the densitometric scans of the autoradiography plates. The relative intensity of the bands in the autoradiogram is registrated against the migration distance in the gel. The arrows indicate the M_r values, estimated from calibration against standard proteins as described in the methods.

amine. This is also the case for the Na^+-dependent 100 kDa band. The sensitivity towards hydroxylamine is a characteristic of acyl phosphates. The 85 kDa phosphoprotein in contrast, is not affected by the hydroxylamine treatment, a behaviour characteristic for phosphoester bonds. The Mg^{2+}-dependent bands at low M_r were also not affected by hydroxylamine (not shown in the figure).

Table I illustrates the time-dependence of the phosphointermediate formation.

TABLE I

TIME DEPENDENCE OF THE FORMATION OF THE PHOSPHOPROTEINS IN A BASAL-LATERAL MEMBRANE PREPARATION

The phosphorylations were done at 0°C. Basal-lateral membranes were present at 1.25 mg/ml. The medium contained 30 mM imidazole-HCl (pH 6.8), 100 mM KCl, 50 μM Ca^{2+} and 1 mM Mg^{2+}. Samples (200 μl) were taken at different time points (time of incubation, t) and the reaction was stopped by addition of 500 μl ice-cold stop solution as described in Methods. The values represent the ratio of peak areas integrated from densitometric scans of the autoradiography plates. To obtain the ratios, the peak areas of the phosphorylation at the different time points, were divided by the peak area of the corresponding band at the first time point (10 s). The values are mean values for two experiments.

M_r ($\times 10^{-3}$)	Ratio of peak areas				
t(s)	10	30	60	120	180
45	1	7	13	22	21
55	1	6	11	16	14
85	1	0.61	0.48	0.32	0.26
100	1	1.1	1.2	1.1	1.2
130	1	1.1	1.1	1	0.9

The ratio of the level of phosphorylation at the different time points to the level of phosphorylation at the first time point (10 s) is given for five different phosphoproteins in a basal-lateral membrane preparation.

The experiment in Table I is done in the absence of La^{3+}, because La^{3+} inhibited the 85 kDa and the Mg^{2+}-dependent bands. As can be seen in the table, the phosphorylation of the Mg^{2+}-dependent bands at M_r 45 000 and M_r 55 000 is relatively slow, and their intensity increases with time.

The 85 kDa band is at the maximum level already before the first time point (10 s) and this intensity decreases with time.

The Ca^{2+}-induced bands at M_r 130 000 and M_r 100 000 finally reach a maximum level of phosphorylation within the first 30 s and this level is maintained during at least 3 min.

In Fig. 4 the effect of lanthanum ions on the Ca^{2+}-induced phosphorylation is compared for three different preparations: renal basal-lateral membranes, erythrocyte inside-out vesicles and sarcoplasmic reticulum of skeletal muscle. It is

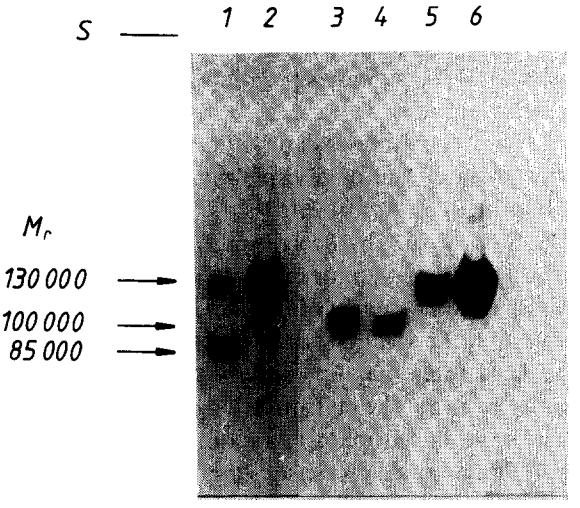

Fig. 4. The effect of lanthanum ions on calcium-dependent phosphorylations. The phosphorylations and analysis on SDS-polyacrylamide gel were done as described in the legend of Fig. 2. The medium contained 30 mM imidazole-HCl (pH 6.8), 100 mM KCl, 50 μM Ca^{2+} and no (lanes 1, 3 and 5) or 100 μM La^{3+} (lanes 2, 4 and 6). The samples were: a Triton X-100 extract of renal basal lateral membranes, 0.15 mg (lanes 1 and 2) sarcoplasmic reticulum from skeletal muscle, 0.4 μg (lanes 3 and 4) and inside-out plasma membrane vesicles from erythrocytes, 0.2 mg (lanes 5 and 6) in a total volume of 200 μl. Note that the 100 kDa band is absent in the renal preparation (lane 1) as is discussed in the text.

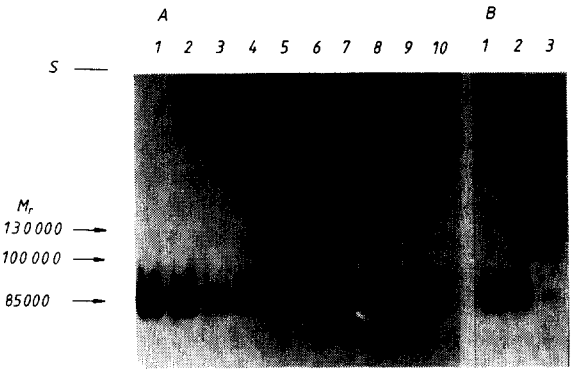

Fig. 5. Autoradiogram of ^{32}P-labeled brush border membrane proteins (part A). For comparison (part B), a preparation of basal-lateral membranes was used in the same experiment (0.26 mg protein). The brush border preparations were: 0.27 mg of a vesicle preparation (part A: lanes 1–6) and 0.14 mg of a Triton X-100 extract from brush border membranes prepared as described in the methods (part A: lanes 7–9). In part A: lane 10 we have used a preparation of purified renal alkaline phosphatase (0.3 μg protein). The phosphorylation was done for 10 s at 0°C. The media contained 30 mM imidazole-HCl (pH 6.8) and 100 mM KCl except in part A lane 5 where KCl was replaced by NaCl. In addition, following solutes were present: Ca^{2+} (50 μM) part A: lanes 2–4, lane 6, lanes 8–10; part B: lanes 2 and 3; EGTA (K^+ salt) (0.5 mM): part A: lanes 1, 5, 7; part B: lane 1; lanthanum (100 μM): part A: lanes 3 and 9, part B: lane 3; Mg^{2+} (1 mM): part A: lane 4; β-glycerophosphate (5 mM) part A: lane 6 and albumin 1.25 mg/ml: part A: lane 10. The total volume, membrane samples included, was 200 μl.

clear that only the 130 kDa band in basal-lateral membranes and in erythrocyte membranes is stimulated by lanthanum. The 100 kDa band in the sarcoplasmic reticulum is slightly inhibited. The renal basal-lateral preparation used here was a Triton X-100 extract prepared as described in Methods. It should be noted that there is no 100 kDa band in this preparation. In most basal-lateral preparations a band at M_r 100000 is observed and the effect of La^{3+} on this band is small. Usually there is a slight inhibition (see Fig. 2, lane 3).

Brush border membranes

An autoradiogram of the phosphorylated proteins of a preparation of brush border membrane vesicles is shown in Fig. 5 part A.

The conditions for the phosphorylations were the same as those used for the basal-lateral membrane vesicles.

The main phosphorylated band has an M_r of 85000 and it comigrates with a preparation of renal alkaline phosphatase (lane 10). The characteristics of this band are exactly the same as for the 85 kDa band in the basal-lateral membrane preparation, e.g. it is abolished in the presence of β-glycerophosphate (lane 6) and inhibited by divalent (lane 4) or trivalent (lane 3, 9) ions at relatively high concentration. Moreover, its intensity is not affected by hydroxylamine (not shown in the figure). There is no indication for phosphorylations in the M_r 100000 or M_r 130000 position when Ca^{2+} or $Ca^{2+} + La^{3+}$ are present (lanes 2 and 3). There is also no phosphorylation in the M_r 100000 position when Na^+ was present in the medium (lane 5). In lanes 7–9 we have used a Triton X-100 extract of the brush border membranes. There is no significant difference with vesicle preparations although in some cases a weak

band at M_r 130000 was detectable in the condition where Ca^{2+} and La^{3+} are both present (lane 9). The intensity of the 85 kDa band is weaker in Triton X-100 extracts as compared to the brush border vesicle preparation. The procedure of Triton X-100 extraction was used, however, to solubilize specifically the $(Ca^{2+} + Mg^{2+})$-ATPase. Other proteins, among which the 85 kDa protein are partially removed by this extraction procedure.

In the presence of Mg^{2+}, analogous to the situation for basal-lateral membranes, there is a phosphorylation in the lower M_r range. This phosphorylation is relatively slow. One band appears at approx. 45 kDa if the incubation time is relatively long (more than 1 min).

In the experiment shown in Fig. 5 however the incubation time was only 10 s and the Mg^{2+}-induced band is not detectable. For comparison in Fig. 5 part B a basal-lateral membrane preparation was used. In the presence of Ca^{2+} and $Ca^{2+} + La^{3+}$, the Ca^{2+}-induced phosphorylations are observed. The 85 kDa band is also present, although weaker than in similar conditions for brush border membranes.

Discussion

In this study we have demonstrated the occurrence of phosphorylated proteins in renal basal-lateral and brush border membranes. The position of these phosphoproteins in the electrophoretograms is compared to that of the phosphoproteins of well characterized preparations such as erythrocyte membrane vesicles, sarcoplasmic reticulum of skeletal muscle and purified renal enzymes, alkaline phosphatase and $(Na^+ + K^+)$-ATPase. As a result an identification of the phosphoproteins can be made.

The calcium requiring phosphorylated band at M_r 130000 can be considered as the phosphorylated intermediate of a $(Ca^{2+} + Mg^{2+})$-ATPase of the 'erythrocyte' type, for the following reasons:

(1) It has a specific requirement for Ca^{2+} versus Mg^{2+}.

(2) It has a specific requirement for ATP versus β-glycerophosphate as a substrate.

(3) It comigrates with the $(Ca^{2+} + Mg^{2+})$-ATPase intermediate from erythrocytes.

(4) It is hydroxylamine sensitive which is a characteristic for an acyl phosphate.

(5) There is a pronounced increase in the intensity of the 130 kDa phosphorylation in the presence of lanthanum ions.

It has been shown that lanthanum increases the steady-state level of phosphorylation in the phosphoprotein of erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase [19,20] probably by decreasing the rate of dephosphorylation of the intermediate in the normal forward running mode of the ATPase. Such in increase in the steady-state level of phosphorylation by lanthanum was not observed for sarcoplasmic reticulum of skeletal muscle [14]. The second calcium-induced phosphorylation at M_r 100000 has the same characteristics as the 130 kDa band except for the stimulation by lanthanum ions. It is therefore conceivable that both bands represent intermediates of a high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase. The relative amount of 100 kDa band versus 130 kDa band varied from preparation to preparation. In some cases the 100 kDa band was not even detectable. In any case, however, the 130 kDa band was the predominant band in the presence of lanthanum ions.

The 100 kDa band could represent the intermediate of a different type of $(Ca^{2+} + Mg^{2+})$-ATPase, e.g. similar to the $(Ca^{2+} + Mg^{2+})$-ATPase of skeletal muscle. This type of $(Ca^{2+} + Mg^{2+})$-ATPase could then be localized in contaminating endoplasmic reticulum vesicles.

On the other hand, it was suggested by Enyedi et al. [27] that for the erythrocyte Ca^{2+}-pump, a 20–40 kDa, calmodulin-binding, regulatory subunit can be cleaved of by mild tryptic digestion of inside-out membrane vesicles. This indicates the possibility that the 100 kDa protein in our preparation could be a proteolytic product of the 130 kDa protein. Graf et al. [32] recently reported about the proteolysis by trypsin of a purified $(Ca^{2+} + Mg^{2+})$-ATPase from human erythrocytes. Limited proteolysis at short times yielded many fragments of various molecular weights. Continued proteolysis, however, results in two trypsin-resistant fragments of M_r 81000 and M_r 33500. No 100 kDa proteolysis product is found as end polypeptide. We have no data about tryptic digestion of the renal $(Ca^{2+} + Mg^{2+})$-ATPase at this moment and the experimental data presented in this study are not sufficient to elucidate the

nature of the 100 kDa band. It should be mentioned in this respect that the situation described here, is very similar to the observations made for smooth muscle membranes [14] and for lymphocyte plasma membranes [21]. In both studies two different Ca^{2+}-requiring phosphoproteins were found. In the latter case evidence was presented for a partial proteolysis of the calcium pump molecule.

In plasma membranes from rat duodenal epithelium [18] there is only one phosphoprotein band that could be identified with the calcium pump protein, at an apparent molecular weight of 115 000–120 000. This phosphoprotein is probably equivalent to the 130 kDa band in our preparation. De Jonge et al. [18] consider their value for the molecular weight as an underestimation since the duodenal $(Ca^{2+} + Mg^{2+})$-ATPase band comigrates with the $(Ca^{2+} + Mg^{2+})$-ATPase of rat heart sarcolemma for which a molecular weight of 150 000 was found [22].

The third band in our basal-lateral membrane preparation at M_r 85 000 displays the characteristics expected for a phosphointermediate of alkaline phosphatase.

(1) It comigrates with purified renal alkaline phosphatase.

(2) The band disappears completely upon addition of 5 mM β-glycerophosphate to the phosphorylation medium. β-Glycerophosphate is a substrate for alkaline phosphatase and competes with ATP.

(3) The 85 kDa band is not affected by hydroxylamine. This is consistent with the properties of a phosphoester bond, e.g. phosphoserine in the case of alkaline phosphatase [23].

In duodenal membrane preparations, two bands with an approximative molecular weight of 84 000 and 64 000 were identified as the phosphointermediates of alkaline phosphatase [18]. The characteristics of these bands are consistent with the 85 kDa band in our preparations. We have also used a commercial preparation of intestinal alkaline phosphatase (Boehringer). The phosphorylated intermediate in this preparation migrates with a somewhat lower M_r value than the kidney enzyme. We have never found a second band at M_r 64 000.

We did not examine the Mg^{2+}-induced lower M_r phosphoproteins in detail. In most experiments these bands were weak because they were phosphorylated too slowly. The slow phosphorylation rate and the fact that these bands were not sensitive towards hydroxylamine treatment, suggests that these phosphoproteins could be the reaction products of protein kinases.

It should be pointed out that the conditions used in this study (short reaction time, temperature 0°C, 6 μM ATP) are not optimal for the phosphorylations mediated by a protein kinase. For the phosphorylation of $(Ca^{2+} + Mg^{2+})$-ATPase, $(Na^+ + K^+)$-ATPase or alkaline phosphatase, the addition of Mg^{2+} was not essential.

The Na^+-induced phosphorylation at M_r 100 000 is also hydroxylamine sensitive (acyl phosphate). This band comigrates with a preparation of purified renal $(Na^+ + K^+)$-ATPase.

The presence of $(Ca^{2+} + Mg^{2+})$-ATPase and $(Na^+ + K^+)$-ATPase in basal-lateral membrane preparations is consistent with the enzymatic and transport data for these membranes.

In brush border membrane preparations, on the other hand, the Ca^{2+}-induced and Na^+-induced phosphoproteins were absent. In Triton X-100 extracts of brush border membranes sometimes a faint band was observed at M_r 130 000 in the presence of Ca^{2+} and La^{2+}. This band can be accounted for by a slight contamination of our brush border preparation with basal-lateral membranes.

The predominant phosphorylation in brush border membranes is the 85 kDa alkaline phosphatase band. The intensity of phosphorylation of this band in purified alkaline phosphatase, brush border membranes and basal-lateral preparations, was roughly proportional to the corresponding enzymatic activity of alkaline phosphatase in these preparations. Alkaline phosphatase is considered as a brush border membrane marker. The presence of the 85 kDa band in our basal-lateral preparations may be due to a relatively large contamination with brush border membranes.

The enzymatic basis of the renal calcium pump is a high affinity $(Ca^{2+} + Mg^{2+})$-ATPase.

The calmodulin stimulation [1], the effect of lanthanum ions on the steady-state level of phosphoprotein and the value for the molecular radius found in this study, are characteristics similar to those found for the red cell calcium pump.

As discussed in a recent review by Schatzmann

[24], the ATP-driven calcium pump at the level of the plasma membrane has been studied extensively in the red cell membrane but exists in many other cell types as well, e.g. in smooth muscle [14]. Moreover, in kidney cells, as in several other cell types, the ATP-driven calcium pump coexists with a Ca^{2+}-extruding Na^{+}-Ca^{2+} exchange system [4]. From micropuncture and microperfusion studies [25] it can be concluded that the Na^{+}-Ca^{2+} exchange pathway is responsible for the bulk of the transepithelial calcium transport. It can be expected that the ATP-driven calcium pump plays a role at very low intracellular calcium concentration. The significance of this ATP-driven calcium pump for transepithelial transport processes remains to be elucidated.

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