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CALCIUM-INDUCED PHOSPHORYLATIONS AND [^{125}I]CALMODULIN BINDING IN RENAL MEMBRANE PREPARATIONS

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Calcium-induced phosphorylated intermediates and calmodulin-binding proteins in membrane preparations from the renal cortex were analyzed by SDS-polyacrylamide gel electrophoresis at low pH, protein electroblotting and [^{125}I]calmodulin overlay. Two calcium-induced phosphoproteins were found, with a molecular mass of 135 and 115 kDa, respectively. By comparing different preparations characterized by marker enzymes, it was shown that the 135 kDa phosphoprotein is localized in the basal-lateral fragment of the plasma membrane, whereas the 115 kDa phosphoprotein is more pronounced in preparations containing a high proportion of endoplasmic reticulum. A prominent calmodulin-binding protein comigrated with the 135 kDa phosphoprotein; there was no calmodulin binding to polypeptides in the molecular mass range of the 115 kDa phosphoprotein. Partial proteolysis by trypsin and the effect of 20 μM La^{2+} on the formation of phosphoproteins before and after trypsinization support the conclusion that the 135 kDa protein can be identified with the plasma membrane calcium pump, whereas the 115 kDa phosphoprotein is the phosphorylated intermediate of a different type of calcium pump probably originating from the endoplasmic reticulum. Calmodulin binding in renal membrane preparations analyzed on Laemmli-type slab gels revealed that there are many calmodulin-binding proteins in our preparations. We have identified one band with the renal calcium pump localized in the basal-lateral membrane. Another calmodulin-binding protein migrating at 108 kDa, is not localized in the basal-lateral membrane and could be one of the calmodulin-binding proteins originating from the cytoskeleton.

Introduction

The formation of a phosphorylated intermediate is well-documented for two different types of calcium pump molecules: the calcium pump of the sarcoplasmic reticulum of skeletal muscle [1,2] and the plasma membrane calcium pump found in red blood cells [3,4]. In a previous paper [5], we have described the formation of two calcium-induced, hydroxylamine-sensitive phosphoproteins in basal-lateral membrane preparations of pig kidney. These phosphoproteins migrated with a molecular mass of approx. 130 and 100 kDa.

Due to the low resolution of the acid SDS-polyacrylamide gels (pH 2.4), the estimation of the molecular radius was not very accurate. It was clear, however, that the 130 kDa protein comigrated with the calcium pump molecule from erythrocytes. We have presented evidence that this 130 kDa protein can be considered as the phosphorylated intermediate of a high-affinity calcium

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

pump localized in the basal-lateral membrane.

Our conclusion is in agreement with recent data, obtained by photoaffinity labeling with ^{125}I-labeled azido-modified calmodulin. It was shown that renal basal-lateral membranes contain a single calmodulin-binding protein which was tentatively identified with the Ca^{2+}-ATPase. The molecular weight was nearly identical to that of the isolated erythrocyte Ca^{2+}-ATPase [6].

The nature of the 100 kDa phosphoprotein observed in renal membrane preparations was not elucidated. We have suggested the possibility that this phosphoprotein is a proteolytic product of the 130 kDa protein or, alternatively, it could be the phosphointermediate of a different type of Ca^{2+}-ATPase (e.g., a calcium pump molecule present in the endoplasmic reticulum). The formation of two different calcium-dependent phosphoproteins was also observed in smooth muscle membranes [7] and in lymphocyte plasma membranes [8]. In the latter case, evidence was presented for a partial proteolysis of the calcium pump molecule.

In the present study, we have used gradient gel electrophoresis in order to improve the resolution of the acid SDS-polyacrylamide gels. The subsequent use of protein blotting and calmodulin overlay techniques allowed the analysis of both phosphoprotein formation and calmodulin binding in identical electrophoretic conditions. Data are reported about the subcellular localization of the phosphoproteins and the calmodulin-binding proteins. The effects of tryptic digestion and La^{2+} on the phosphoprotein formation is described.

Materials and Methods

Membrane preparation

Pig kidneys were perfused with an ice-cold isotonic sucrose buffer (250 mM sucrose/10 mM triethanolamine-HCl (pH 7.6)/0.1 mM PMSF). The cortex was dissected, minced and stored at $-70\,^{\circ}C$.

A membrane preparation enriched in the basal-lateral segment of the plasma membrane was obtained following the procedure described by Sacktor et al. [9] for rat-kidney cortex, with some modifications. Pig kidney cortex (25 g) was thawed in sucrose buffer. Homogenization was performed with a loose-fitting Teflon-glass homogenizer for 15 strokes at 700 rpm and subsequently with an Ultraturrax (type TP 18-10 Janke and Kunkel IKA-Werke) at setting 9, three times for 30 s.

The centrifugation steps involved in the preparation of crude plasma membranes were performed in a Sorvall GSA-rotor. For the purification of the crude plasma membranes, a self-generating Percoll gradient of 10% Percoll (Pharmacia Fine Chemicals) in sucrose buffer was used. The centrifugation was performed in a Beckman rotor (type 60 Ti) at 15 800 rpm (25 000 × *g*) for 20 min. The procedure used to remove the Percoll was as described in Ref. 9, except that the buffer solution used here contained 30 mM imidazole-HCl (pH 7.0), 100 mM KCl, 100 mM sucrose and 5 mM Tris-azide. The final pellet was also suspended in this buffer at about 5 mg/ml.

The second supernatant, after a centrifugation at 12 250 rpm in a Sorvall GSA-rotor (24 000 × *g*), is normally discarded in the procedure for the preparation of crude plasma membranes. We have saved this supernatant and used it as the starting material for the preparation of renal microsomes. The preparation of renal microsomes, enriched in marker enzymes for the endoplasmic reticulum was obtained following the method described by Landon and Norris [10]. The 24 000 × *g* supernatant saved from the crude plasma membrane preparation is centrifuged at 17 500 rpm for 40 min in a Sorvall SS-34 rotor (37 000 × *g*). The pellet is discarded and the supernatant is pelleted at 30 000 rpm for 2 h in a Kontron TFT 45 rotor (105 000 × *g*). the final pellet is resuspended in the same buffer as used for the suspension of the basal-lateral membranes. Renal brush-border membrane vesicles were prepared as described elsewhere [11].

Alkaline phosphatase was purified from porcine kidney according to Mössner et al. [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 $-70\,^{\circ}C$.

Protein was determined by the method of Lowry et al. [14] after precipitation of the membrane protein by 10% (w/v) ice-cold trichloroacetic acid. The membrane preparations were assayed for marker enzymes as described previously [5]. The enrichment factors versus the homogenate for the

crude plasma membranes, Percoll-purified basal-lateral membranes and renal microsomes, are summarized in Table II.

Phosphorylation experiments and acid gradient gel electrophoresis

The phosphorylation reactions were performed exactly as described elsewhere [5]. The reaction time was 10 s on ice, the composition of the reaction media is indicated in the legends to the figures. The phosphorylated intermediates of ion-transport ATPases are acyl-phosphates and labile in alkaline medium. The analysis of these phosphoproteins on SDS-polyacrylamide gel electrophoresis requires acid buffers. In our previous work, we have used a polyacrylamide slab gel (0.75 mm thick, 5% acrylamide) electrophoresis at pH 2.4. A better resolution can be obtained by using a polyacrylamide gel electrophoresis with discontinuous buffers in the acid range, as described by Amory et al. [15], or as was done in this study, by using an acid gradient gel. We have used the acid gel described by Lichtner and Wolf [16] in the form of a gradient ranging from 4 to 15% acrylamide.

In order to obtain optimal separation in the high molecular weight range, the gradients were made concave-shaped. This was achieved by using an open chamber gradient mixer with a smaller (2 cm diameter) mixing chamber containing the high density medium and a larger (3 cm diameter) reservoir chamber containing the low density medium. The high density medium was 22.7 ml of a solution containing 15% (w/v) sucrose, 15% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, 0.00025% (w/v) $FeSO_4 \cdot 7H_2O$, 0.1% (w/v) ascorbic acid and 0.03% (w/v) H_2O_2 in electrophoresis buffer.

The low density medium was 50.9 ml of an identical solution without the sucrose, acrylamide and bisacrylamide. The electrophoresis buffer adjusted to pH 2.4 contained 93.8 mM citric acid, 12.4 mM phosphoric acid, 12 mM Tris-base and 0.1% (w/v) sodium dodecyl sulfate.

Two identical gels with a total volume of 34 ml were poured by using a two-channel pump (LKB Microperpex 2132).

Protein electroblotting and calmodulin overlay

Electrophoretic transfer of proteins from the acid gradient gels to nitrocellulose sheets (Millipore HAWP 304 FO) was performed according to the method described by Bittner et al. [17]. Immediately after electrophoresis, the gel was equilibrated for 30 min in 500 ml of a transfer buffer (25 mM sodium phosphate, pH 6.5). Electroblotting was conducted for 16 h at 15 V (0.3 A) in the Bio-Rad Trans Blot Cell, thermostated at 4°C. In experiments with Laemmli-type slab gels [18], the transfer buffer was a solution containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% (w/v) methanol, and electroblotting was conducted for 16 h at 30 V (0.1 A) as described by Wuytack et al. [19].

After blotting, the part of the blot containing the lanes with phosphorylated proteins was cut off, dried and used for autoradiography on Kodak X-omat R film (XR-1). The part of the blot containing lanes with unlabeled proteins was incubated in a quench solution in order to saturate the protein-binding sites prior to calmodulin overlay. The incubation was done with constant agitation at 37°C in a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 mM $CaCl_2$, 0.5% (w/v) casein and 0.5% (w/v) bovine serum albumin.

Subsequently, the blot was incubated in 10 ml quenching buffer, containing 0.3 nmol [^{125}I]calmodulin ($5 \cdot 10^7$ cpm/nmol), during 30 min at room temperature. This was followed by a washing procedure to remove the unbound radioactivity. The blot was washed, then dried and autoradiographed. A detailed description of this method will appear in a study of the Ca^{2+}-transport ATPases in smooth muscle membranes (Wuytack, F., Raeymaekers, L., Verbist, J., De Smedt, H. and Casteels, R., unpublished data).

Calmodulin was purified from bovine brain [20]. Iodination was performed using a commercial radioiodination kit. The specific activity was about $5 \cdot 10^7$ cpm/nmol of calmodulin. The iodinated product was stored at −20°C in an aqueous solution containing 2% bovine serum albumin.

The iodination kit (NEZ 151) and [γ-^{32}P]ATP (NEG-002A) were obtained from New England Nuclear.

For a quantitative analysis of the data, the

labeled protein bands detectable on the autoradiograph were cut out the blot and the radioactivity within this band was counted in a liquid scintillation counter (Packard Tri-carb 300 C).

Calibration proteins (Boehringer Combithek 750115) were used for estimation of M_r. The part of the blot with the M_r reference proteins was cut off and stained with India Ink as described by Hancock and Tsang [21]. M_r values were obtained from curves relating M_r values of standard proteins to their corresponding R_F values.

Results

In the experiment, illustrated in Fig. 1, the formation of calcium-dependent phosphoproteins and the binding of [^{125}I]calmodulin are compared for different preparations in identical electrophoretic conditions.

Part A of the figure shows the result for a basal-lateral membrane preparation. The lanes 1–5 contain the phosphorylated proteins. It is evident that in the presence of 50 μM calcium (lane 2), two phosphoproteins are formed at 135 and 115 kDa, respectively. These phosphoproteins are not formed in the presence of 5 mM EGTA (lane 1).

The effect of La^{2+} (20 μM) on the Ca^{2+}-dependent phosphorylation is shown in lane 3. The formation of the 135 kDa phosphoprotein is largely stimulated. From the quantitative data shown in

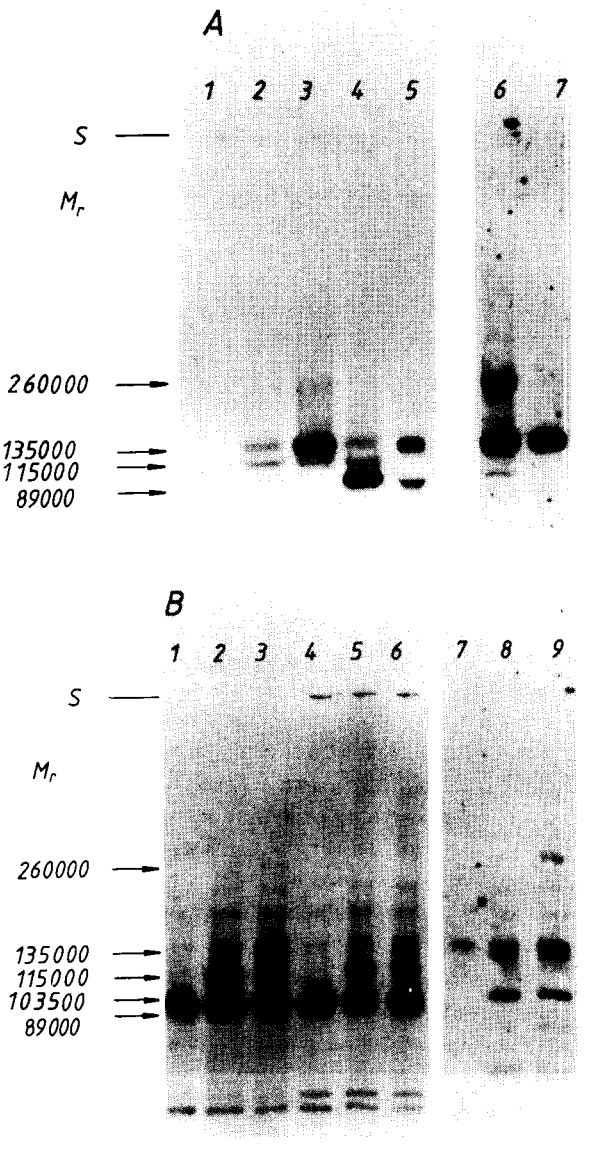

Fig. 1. Autoradiograms of nitrocellulose electroblots containing ^{32}P-labeled and [^{125}I]calmodulin-labeled proteins. Part A, lanes 1–5 and part B, lanes 1–6 are phosphoproteins. Part A, lanes 6–7 and part B, lanes 7–9 are [^{125}I]calmodulin-binding proteins. Phosphorylation was carried out for 10 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 7 at room temperature) and 100 mM KCl. In addition, the following solutes were present: EGTA (K^+-salt), 5 mM: part A, lane 1 and part B, lanes 1 and 4; Ca^{2+}, 50 μM: part A, lanes 2–5 and part B, lanes 2, 3, 5 and 6; La^{3+} (20 μM): part A, lane 3 and part B, lanes 3 and 6; β-glycerophosphate, 5 mM: part A, lanes 1–3. The total volume, including the samples, was 200 μl. The samples were 0.25 mg basal-lateral membranes in part A, 1–4; 0.23 mg erythrocyte plasma membranes plus 0.16 μg of a preparation of purified alkaline phosphatase in part A, lane 5; 0.37 mg of a renal crude plasma membrane preparation in part B, lanes 1–3; and 0.27 mg of a preparation of renal microsomes in part B, lanes 4–6. The samples for the calmodulin overlay were precipitated in the same stop solution as used to stop the phosphorylation and resuspended in the sample buffer. These samples contained 0.25 mg basal-lateral membranes in part A, lane 6; 0.09 mg erythrocyte plasma membranes in part A, lane 7 and part B, lane 7; 0.27 mg renal microsomes in part B, lane 8; and 0.37 mg of a renal crude plasma membrane preparation in part B, lane 9. The stop solution, washing procedure, sample buffer and electrophoresis conditions were as described elsewhere [7]. Subsequently, the proteins on the electropheretogram were electroblotted. The part of the blot containing the unlabeled samples was cut off and treated with [^{125}I]calmodulin as described in Materials and Methods. In some control experiments, the incubation buffer used for calmodulin overlay contained no Ca^{2+} but 5 mM EGTA. In this case, no calmodulin binding was detected.

TABLE I

RELATIVE AMOUNTS OF CALCIUM-INDUCED PHOSPHORYLATION AND THE EFFECT OF LANTHANUM IONS

The phosphorylations were performed as described in the legend of Fig. 1. The values represent the ratios of peak areas integrated from densitometric scans of the autoradiography plates, made by means of the LKB 2202 Ultrascan Laser Densitometer. To obtain the ratios, the peak area of the phosphorylation band at 135 kDa was divided by the peak area of the phosphorylation band at 115 kDa in the same preparation. The effect of La^{3+} was obtained by dividing the peak areas measured in the presence of 20 μM La^{3+} by the corresponding peak areas measured in the absence of La^{3+}. All values are mean values for three different preparations.

Preparation	Ratio of peak areas (135 kDa/115 kDa)		Effect of lanthanum (peak area (20 μM La^{3+})/peak area (no La^{3+}))	
	no La^{3+}	20 μM La^{3+}	135 kDa	115 kDa
Crude plasma membranes	0.25	1.14	3.55	0.8
Basal-lateral membranes	1.06	10.36	5.55	0.56
Renal microsomes	0.24	0.58	1.6	0.66

Table I, it can be concluded that the formation of the 115 kDa phosphoprotein is slightly reduced.

Lane 4 is identical to lane 2, except that no β-glycerophosphate is present. In this case, there is formation of a strong phosphoprotein band at 89 kDa.

In lane 5, we have used a mixture of inside-out vesicles from erythrocytes and a sample of purified renal alkaline phosphatase. In the erythrocyte membranes, there is only one calcium-dependent phosphoprotein at 135 kDa. The 89 kDa phosphoprotein is the intermediate formed in the alkaline phosphatase preparation. In our previous work [5], we have pointed out that the 89 kDa phosphoprotein is not an acyl phosphate (hydroxylamine-insensitive) and that its presence in renal preparations is due to contaminating brush-border membranes. The data shown in lanes 1–5 are in full agreement with our previous data. In this experiment, however, a better resolution and a more accurate molecular weight determination are obtained by using a gradient gel instead of a homogeneous gel.

In lanes 6 and 7, the second part of the blot is shown. This part of the blot contains no phosphorylated proteins, it was cut off and treated for [^{125}I]calmodulin binding as described in Materials and Methods. We can detect only one calmodulin-binding protein in the erythrocyte membrane preparation (lane 7) which has the same molecular weight value (135 000) as the calcium-dependent phosphoprotein in erythrocyte membranes (lane 5).

In the renal membranes (lane 6), several calmodulin-binding proteins can be observed. One of these is a 135 kDa protein which comigrates with the upper phosphorylation band in lanes 2–4. In renal membranes, a second calmodulin-binding protein is found at M_r of about 260 000, and a weaker band at 103 000. It is important to note that we could detect no calmodulin binding at 115 000 which is the molecular weight of the lower calcium-induced phosphoprotein band.

In part B of Fig. 1, similar observations can be made for two other renal preparations. The phosphoprotein formation in a crude plasma membrane preparation (lanes 1–3) and in a microsomal fraction (lanes 4–6) is shown. The calmodulin binding is shown in lanes 7 (erythrocyte plasma membranes), 8 (renal microsomes) and 9 (renal crude plasma membranes). The 115 kDa phosphoprotein is the prominent band in both renal crude plasma membranes (lane 2) and renal microsomes (lane 5).

In the presence of 20 μM La^{3+}, the amount of 135 kDa phosphoprotein is increased in renal crude plasma membranes (lane 3) and to a lesser extent in renal microsomes (lane 6). Lanes 1 and 4 contain membranes phosphorylated in the presence of EGTA and show the calcium-independent phosphorylations.

A calmodulin-binding protein at 135 kDa is found in both renal preparations (lanes 8 and 9) and comigrates with the 135 kDa protein in erythrocytes (lane 7). In the renal preparations, a few other calmodulin-binding proteins are ob-

served (e.g., at 260 and 103 kDa), but we could detect no calmodulin binding in the 115 kDa position.

A quantitative evaluation of the relative amount of phosphoprotein formed at 135 kDa and 115 kDa in the three renal preparations is shown in Table I. The ratio of the amount of phosphoprotein 135 kDa/115 kDa is given in the presence and in the absence of lanthanum ions. The ratio is high for the basal-lateral membranes but low for the microsomes. This is much more pronounced in the presence of La^{3+}. In this case, the ratio is 10.36 for basal-lateral membranes but the 115 kDa protein remains the most prominent band for renal microsomes (ratio 0.58). The effect of La^{3+} on the 135 kDa and on the 115 kDa phosphoprotein band is also shown in Table I.

In the experiment described in Fig. 1, gel electrophoresis at low pH was used in order to compare phosphorylated intermediates and calmodulin-binding proteins in identical electrophoretic conditions. A better evaluation of the calmodulin binding can be obtained by using SDS-polyacrylamide gel electrophoresis with discontinuous buffers. In Fig. 2, the result of a calmodulin overlay using a disc gel of the Laemmli-type is shown. In this experiment, the different samples were suspended in the sample buffer described in the Laemmli procedure [18], but the solubilization was performed at 37 °C for 15 min. The amount of protein was kept at 100 μg per lane for all samples, except for the erythrocyte membranes where 28 μg was used.

In these conditions, much more calmodulin-binding proteins are detected. A calmodulin-binding protein at 135 kDa is present in all preparations. It is very pronounced in erythrocyte plasma membranes (Fig. 2, lane 5). In the figure, we have compared different membrane preparations from renal cortex: the homogenate in lane 1, a crude plasma membrane preparation in lane 2, a purified basal-lateral preparation in lane 3 and a microsomal fraction in lane 4. In lane 6, finally, a preparation of brush-border membrane vesicles was used.

It can be observed that for the renal preparations, the amount of calmodulin binding in the 135 kDa position is roughly proportional to the degree of purification with respect to the basal-lateral

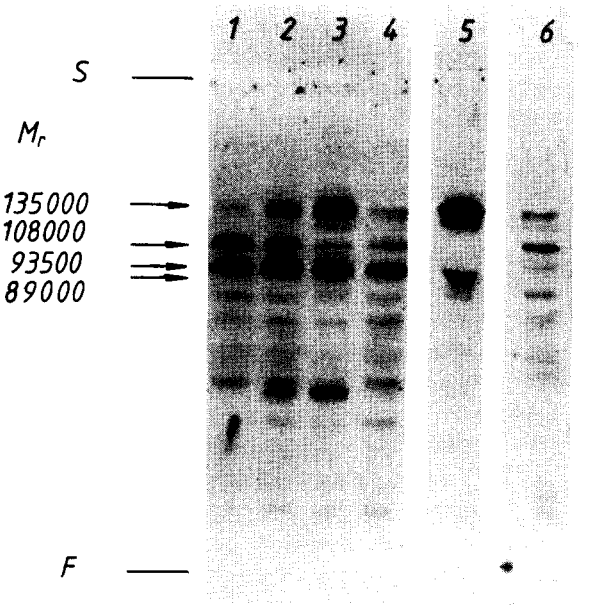

Fig. 2. Autoradiogram of a nitrocellulose electroblot containing [^{125}I]calmodulin-binding proteins. A Laemmli-type slab gel electrophoresis was done on 0.75 mm thick slab gels with a stacking gel of 3% and a resolving gel of 7.5% acrylamide. Proteins were dissolved in 2% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), 1% mercaptoethanol, 0.03% Bromophenol blue and incubated for 15 min at 37 °C. The samples applied to the gel were 40 μl of this mixture containing 100 μg of protein, except in lane 5 where the sample contained only 28 μg protein. The sample in lane 1 contains the homogenate of renal cortex, lane 2 is a renal crude plasma membrane preparation, lane 3 is a purified preparation of basal-lateral membranes, lane 4 is a renal microsomal preparation, lane 5 is a sample of erythrocyte plasma membranes and lane 6 is a preparation of renal brush-border membrane vesicles. Electroblotting on nitrocellulose membranes, [^{125}I]calmodulin overlay, molecular weight determination and autoradiography were as described in Materials and Methods.

segment of the membrane. For another calmodulin-binding protein at 108 kDa, the inverse is found. The band is very pronounced in the homogenate (lane 1) and is very weak in the purified basal-lateral membranes (lane 3). It can be seen in lane 6 that the 108 kDa protein is the most prominent calmodulin-binding protein band in a preparation of brush-border membrane vesicles. Several other calmodulin-binding proteins, e.g., at 121, 93, 74 and 54 kDa can be observed in all preparations.

TABLE II

ENZYME ACTIVITIES AND CALMODULIN-BINDING CHARACTERISTICS

The values represent the specific activities (mean ± S.E. (number of preparations)) found in the homogenate and the enrichment factors (mean ± S.E. (number of preparations)) found in three different renal membrane preparations. Enzyme activities are expressed in U/mg protein and were determined as described elsewhere [5]. The [^{125}I]calmodulin binding to the 135 kDa protein is also given for the three preparations in the form of the relative amount of binding in the preparation versus the amount of binding in the homogenate. The amount of calmodulin binding was determined by counting the labeled protein bands in a liquid scintillation counter.

Preparation	($Na^+ + K^+$)-ATPase	Aminopeptidase M	NADH-cytochrome *c* oxidoreductase	Cytochrome *c* oxidase	[^{125}I]calmodulin binding at the 135 kDa protein
Homogenate	0.087 ± 0.01 (8) [a]	0.165 ± 0.007 (8) [a]	0.212 ± 0.01 (7) [a]	0.239 ± 0.019 (6) [a]	1066 ± 198 (3) [b]
Enrichment factor	1	1	1	1	1
Crude plasma membranes	4.3 ± 1.3 (8)	2.2 ± 0.1 (8)	1.4 ± 0.1 (7)	1.6 ± 0.1 (6)	1.72 ± 0.14 (3)
Basal-lateral membranes	14.5 ± 2.5 (11)	1.6 ± 0.1 (11)	0.7 ± 0.1 (10)	0.2 ± 0.03 (9)	4.25 ± 0.53 (3)
Microsomes	0.6 ± 0.1 (4)	2.1 ± 0.3 (4)	2.0 ± 0.2 (3)	0.2 ± 0.1 (3)	1.68 ± 0.23 (3)

[a] Specific activity in U/mg.

[b] Value is cpm found in the 135 kDa band per mg protein applied to the gel. The incubation buffer contained 30 nM [^{125}I]calmodulin with a specific activity of $5 \cdot 10^7$ cpm/nmol.

In Table II, the enzymatic data for the renal preparations are summarized and compared with the calmodulin binding. The data are expressed in the form of the enrichment factor versus the homogenate. The ratio of the amount of [^{125}I]calmodulin binding at 135 kDa in each fraction versus the amount of binding in the homogenate is also given in this table.

In Fig. 3, the effect of a trypsinization procedure on the subsequent formation of phosphorylated intermediates is shown. Renal basal-lateral membranes (lanes 1–5) are compared to plasma membranes from erythrocytes (lanes 6–9). trypsinization was induced by incubation of the samples during 45 min on ice with 45 μg/ml trypsin (Sigma No. T-8253). The trypsinization reaction was stopped by addition of an excess of trypsin inhibitor (285 μg/ml final concentration).

In the conditions where no trypsinization was required, the same incubation procedure was followed, but trypsin inhibitor was added before trypsin. Subsequently, La^{3+} was added and the phosphorylation procedure was started. For the renal samples, 5 mM β-glycerophosphate was present in order to suppress the formation of the intermediate of alkaline phosphatase (still detectable as a weak band at 89 kDa).

For the erythrocyte plasma membranes, there is only one single calcium-induced phosphoprotein band before trypsinization. The M_r value of this phosphoprotein is shifted by the trypsinization to about 115 000–120 000 (lanes 8 and 9). It is also evident in the figure, that the stimulatory effect of La^{3+} on the phosphoprotein formation is still preserved for the tryptic digestion product.

The situation in the case of the renal membrane preparation is more complex. The 135 kDa phosphoprotein (lanes 2 and 3) is affected by trypsin in the same way as observed for the erythrocyte phosphoprotein. The M_r value for this calcium-induced and lanthanum-stimulated phosphoprotein is shifted to about 115 000–120 000 (lanes 3 and 4).

The second Ca^{2+}-induced phosphoprotein at 115 kDa is also affected by the trypsinization. Due to the downward shift of the 135 kDa protein, the amount of phosphoprotein in the 115 kDa position after trypsinization is difficult to evaluate. In lanes 4 and 5, however, there is clear evidence for a new 55 kDa phosphoprotein after trypsinization. The effect of La^{3+} on the original 115 kDa protein (La^{3+} induces a slight inhibition) is also preserved for the trypsinization product at 55 kDa (lanes 4

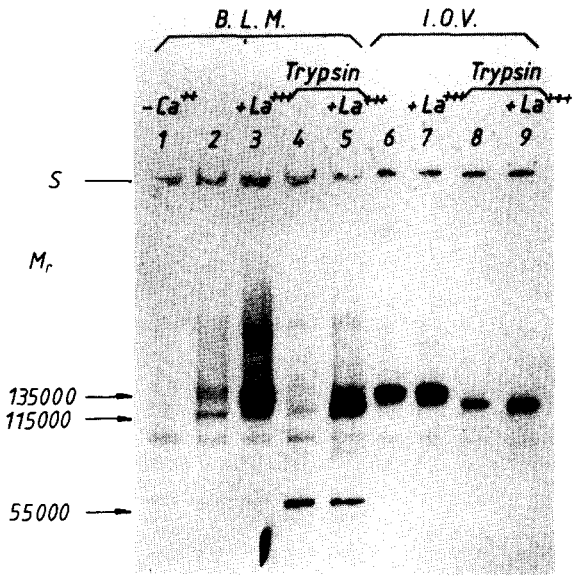

Fig. 3. Autoradiogram of a nitrocellulose electroblot containing the phosphoproteins obtained by phosphorylation of control samples and trypsinized samples. Trypsinization was started in a medium containing all components of the phosphorylation medium, except La^{3+}. Trypsinization occurred at 0 °C for 45 min. The final concentration of trypsin was 45 μg/ml. The reaction was stopped by addition of trypsin inhibitor (285 μg/ml final concentration). In the conditions where no trypsinization was required, trypsin inhibitor was added first before trypsin. Subsequently, La^{3+} was added, where needed, and the phosphorylation was started by addition of [γ-^{32}P]ATP (6 μM final concentration at 13 Ci/mmol). The final composition of the phosphorylation media was: 30 mM imidazole-HCl (pH 7 at room temperature); 100 mM KCl, and in addition, 5 mM EGTA in lane 1 and 50 μM Ca^{2+} in the other lanes; 5 mM β-glycerophosphate in lanes 1–5 and 20 μM La^{3+} in lanes 3, 5, 7 and 9. The phosphorylation medium had a final volume of 200 μl and the total amount of protein was 0.26 mg basal-lateral membranes (B.L.M.) in lanes 1–5 and 0.186 mg erythrocyte plasma membranes (I.O.V.) in lanes 6–9. Phosphorylation, electrophoresis, electroblotting, molecular weight determination and autoradiography were as described in the legend of Fig. 1 and in Materials and Methods.

and 5). In preparations where a higher amount of 115 kDa protein was present (renal microsomes), additionally, a weak band at about 35 kDa was also detected (not detectable in Fig. 3).

The 55 kDa phosphoprotein observed here, comigrated with a phosphoprotein band obtained after trypsinization of a membrane preparation of sarcoplasmic reticulum from skeletal muscle and measured in the same conditions (Wuytack, F., Raeymaekers, L., Verbist, J., De Smedt, H. and Casteels, R., unpublished data).

Discussion

Identification of the calcium-induced phosphoproteins in renal membranes

The M_r values found for the two calcium-induced phosphoproteins in the present study are somewhat higher than our earlier values [5]. There is, however, good agreement concerning the fact that one of the phosphoproteins in the renal preparations migrates with the same M_r value as the calcium-induced phosphoprotein in erythrocyte plasma membranes, whereas the second phosphoprotein migrates with the same M_r value as the calcium-induced phosphoprotein in sarcoplasmic reticulum of skeletal muscle. The use of gradient gel electrophoresis, provides better resolution of the two phosphoproteins and the use of protein electroblotting and overlay techniques made it possible to compare phosphoprotein formation and [^{125}I]calmodulin binding in identical electrophoretic conditions.

In our previous work [5], we have identified the 135 kDa protein as the phosphorylated intermediate of a plasma membrane calcium pump of the 'erythrocyte' type. In this paper, we have presented several indications that the second calcium induced phosphoprotein at 115 kDa is derived from the endoplasmic reticulum.

(1) In Table I, three different renal membrane preparations are compared. Both calcium-induced phosphoproteins are present in each preparation. However, the ratio of the amount of phosphoprotein formed in the two phosphorylation bands (135 kDa/115 kDa) is very different for the three preparations. The difference becomes very pronounced if the phosphorylations are performed in the presence of 20 μM La^{3+}. In this condition, the basal-lateral membrane preparation had 10-times more phosphoprotein at 135 kDa than at 115 kDa. For the renal microsomes, on the other hand, the amount of 135 kDa phosphoprotein is comparatively low even in the presence of La^{3+}, and the 115 kDa phosphoprotein is the prominent band. The results suggest a basal-lateral localization for the 135 kDa protein but a different localization for the 115 kDa phosphoprotein.

The 135 kDa phosphorylation is stimulated by La^{3+} up to 5-fold in preparations containing a high proportion of basal-lateral membranes. In renal microsomes, on the other hand, the La^{3+} effect is relatively small (1.6-fold stimulation). This can be due to the fact that in determining the La^{3+} stimulation, we did not substract the 'background' level of phosphoprotein. The calcium-dependent and calcium-independent background phosphorylation is relatively more important for the microsomal fraction.

For the 115 kDa phosphoprotein, 20 μM La^{3+} reduces the amount of phosphorylation by 20–40% in all preparations.

(2) A second indication is found from comparison of the molecular mass of the calcium-induced phosphoproteins with the molecular mass of the calmodulin-binding proteins measured in identical conditions on the same gel. It is observed that one of the [^{125}I]calmodulin-binding proteins migrates with exactly the same molecular mass as the 135 kDa phosphoprotein. Equally important however, is the observation that there was no [^{125}I]calmodulin binding at the level of the 115 kDa phosphoprotein. The same observation was made for crude plasma membrane preparations and for microsomes (Fig. 1B), where the amount of 115 kDa phosphoprotein is much more pronounced than in the basal-lateral preparation. Controlled proteolysis of the purified Ca^{2+}-ATPase of the erythrocyte membrane [22] leads to the formation of a calmodulin-binding fragment of about 90 kDa. The ability to bind calmodulin is destroyed by a further proteolytic step converting the 90 kDa polypeptide to a 81 kDa fragment. If the proteolytic pattern found for the purified erythrocyte ATPase is applicable for the renal plasma membrane ATPase, it becomes very unlikely that the 115 kDa protein is a proteolytic product derived from the original plasma membrane ATPase. The observation that there was no [^{125}I]calmodulin binding to a polypeptide with the same M_r value as the 115 kDa phosphoprotein, rather suggests that this phosphoprotein is the intermediate of a Ca^{2+}-ATPase of a different type. The lack of response to calmodulin is one of the differences between the red cell Ca^{2+} pump and the sarcoplasmic reticulum Ca^{2+} pump [23]. In the experimental conditions described in Fig. 1, there was no [^{125}I]calmodulin binding to the proteins of a preparation of sarcoplasmic reticulum from skeletal muscle (data not shown in the figure).

(3) A third indication for the origin of the 115 kDa protein follows from the trypsinization experiment described in Fig. 3. The partial trypsinization of the Ca^{2+}-ATPase in erythrocyte membranes as shown in lanes 6–9 is in complete agreement with the observations made by Zurini et al. [22] for purified erythrocyte ATPase. The authors describe the formation of a single phosphoprotein band at $M_r = 120\,000$ if the pH was kept acid in order to preserve the acylphosphate bond. The same behavior is observed in lanes 6–9 for a sample of erythrocyte plasma membranes. It is also evident from our observations in Fig. 3 that the stimulation of the phosphoprotein formation by 20 μM La^{3+}, which is a characteristic of the original protein, is still preserved for the tryptic product at 120 kDa.

A similar behavior is found to the 135 kDa band in the renal preparations. The original phosphoprotein is shifted by trypsin to the 120 kDa position and the stimulatory effect of La^{3+} is preserved.

The fact that the second Ca^{2+}-induced phosphoprotein in the renal preparations has a molecular mass (115 kDa) which is very close to the tryptic product of the 135 kDa phosphoprotein, complicates the observations in this M_r range.

The 115 kDa phosphoprotein, however, is not stimulated but slightly reduced by 20 μM La^{3+}.

After trypsinization, there is formation of a new phosphoprotein at $M_r = 55\,000$. The phosphorylation of this 55 kDa protein is also slightly inhibited by 20 μM La^{3+}. This suggests that the 55 kDa phosphoprotein is a proteolytic product derived from the 115 kDa phosphoprotein. In renal microsomes, where a larger amount of the 115 kDa phosphoprotein is present, trypsinization leads to the formation of a strong phosphorylation band at 55 kDa and a much weaker band at about 35 kDa. The formation of these two digestion products is very similar to what is observed for the Ca^{2+}-ATPase from sarcoplasmic reticulum [24].

Calmodulin-binding proteins in renal membranes

Photoaffinity-labeling of renal basal-lateral plasma membranes with ^{125}I-labeled azido-mod-

ified calmodulin was recently described [6]. In this study, we describe the application of a [^{125}I]calmodulin overlay technique to label the calmodulin-binding proteins. A similar technique has been used for the detection of calmodulin-binding proteins in intestinal brush-border and microvillar preparations [25,26]. Recently, a combination of [^{125}I]calmodulin overlay and ^{32}P-phosphoenzyme electrophoresis was also used for the molecular weight determination of the Ca^{2+}-ATPase in synaptosomal plasma membranes [27]. We have used two different types of gels. The acid gradient gel was intended to compare phosphoprotein formation and [^{125}I]calmodulin binding; the Laemmli-type gel has the advantage of better resolution. The results obtained for both gel types (Figs. 1 and 2) are not identical. In the conditions used for the acid gels, only three calmodulin-binding proteins are detected. the molecular weights are 260 000, 135 000 and 103 000. Proteins in the low molecular weight range can eventually be missed, because, in order to obtain optimal separation in the molecular weight range around 115 000–135 000, the duration of the electrophoresis was increased and the front migrated off the gel.

As already discussed, the 135 kDa protein comigrated with one of the Ca^{2+}-induced phosphoproteins and was identified with the plasma membrane Ca^{2+}-ATPase. Our data do not allow identification of the other calmodulin-binding proteins. The 103 kDa protein is relatively more pronounced in crude preparations than in purified basal-lateral membranes.

The 260 kDa band, on the other hand, is very strong in the basal-lateral preparation. The M_r value suggests that this band could represent the dimer of the 135 kDa protein. On the other hand, several high molecular weight calmodulin-binding proteins of cytoskeletal origin are described in various cell types (e.g., intestinal cells [25,26], erythrocytes [28], brain [29–31]). The presence of similar proteins in our preparations can certainly not be excluded.

In the conditions used for sample treatment for the Laemmli-type gels, high molecular weight calmodulin-binding proteins were not detected. The prominent band with this type of gel is the 135 kDa protein, which is also very pronounced in erythrocyte plasma membranes. The identification of this band with the renal calcium pump molecule, is supported by the relative amount of calmodulin binding found in this position for different preparations, versus the amount of binding in the homogenate. It can be concluded from the data in Table II that the [^{125}I]calmodulin binding in the 135 kDa band is roughly proportional to the enrichment factor for $(Na^+ + K^+)$-ATPase in the preparation. It should be pointed out, however, that the [^{125}I]calmodulin overlay is not a quantitative technique. The procedure required at least partial renaturation of an originally SDS denatured polypeptide, and the efficiency of renaturation is not known.

For the other calmodulin-binding proteins at lower molecular mass, no identification can be made. However, the 108 kDa protein is clearly not localized in the basal-lateral membrane. The amount of this calmodulin binding is higher in the homogenate than in the membrane preparations. The 108 kDa protein is the prominent calmodulin-binding protein in a preparation of brush-border membrane vesicles. One of the major proteins, solubilized from the chicken intestinal microvillus is a calmodulin-binding protein of similar molecular weight (105 000–110 000) [32]. Although we have made no attempt to characterize the 108 kDa protein in our preparations, it could be one of the calmodulin-binding proteins originating from the cytoskeleton.

Comparison of the renal preparations with erythrocyte membranes, suggests that some of the calmodulin-binding proteins (e.g., at 121 and 93 kDa) can be attributed to proteolytic products of the original calcium pump molecule. As already discussed, the calmodulin-binding ability is preserved in fragments larger than 90 kDa.

From our data the relative amount of calmodulin-binding proteins cannot be estimated. The presence of large amounts of calmodulin-binding proteins, for example, in the cytoskeleton, could therefore complicate the purification of the renal high-affinity calcium pump by a calmodulin-affinity gel.

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