CALMODULIN STIMULATION OF RENAL (Ca^{2+} + Mg^{2+})-ATPase

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Received 26 May 1981

1. Introduction

There is evidence for at least two different transport systems for Ca^{2+} in basal–lateral plasma membranes of proximal tubular epithelial cells: a Na^+-gradient-driven Na^+/Ca^{2+} exchange and an ATP-driven transport system [1].

An ATP-driven Ca^{2+} uptake is also present in renal microsomes [2]. In a variety of tissues a (Ca^{2+} + Mg^{2+})-ATPase corresponding to the Ca^{2+} transport has been demonstrated (e.g. in skeletal muscle [3], erythrocytes [4], adipocyte plasma membranes [5], smooth muscle [6] and rat duodenum [7]).

The presence of a (Ca^{2+} + Mg^{2+})-ATPase in renal preparations was mentioned in [2], but the authors do not give quantitative information. Ca^{2+}-ATPase activity was reported in renal basal–lateral membranes [8], but it was measured in the absence of Mg^{2+} and is stimulated by Ca^{2+} in the millimolar range. As discussed in [9] this Ca^{2+}-ATPase is probably not correlated with Ca^{2+} transport.

In order to demonstrate the (Ca^{2+} + Mg^{2+})-ATPase activity in the conditions for Ca^{2+} transport, we enriched a renal membrane preparation with (Ca^{2+} + Mg^{2+})-ATPase by detergent solubilization and mixed-micelle gel chromatography. This procedure was originally described for the purification of (Ca^{2+} + Mg^{2+})-ATPase from erythrocytes [10]. By this procedure the high 'background' activity of Mg^{2+}-ATPase in our preparation is reduced, so that Ca^{2+} stimulation and the effect of calmodulin become apparent.

2. Experimental

A crude preparation, enriched in basal–lateral plasma membranes, was obtained by differential centrifugation according to [11] from hog kidney cortex. To have enough starting material we omitted the free flow electrophoresis described by these authors. The membranes obtained after the centrifugation steps were suspended in 5 ml of buffer medium containing: 50 mM imidazole–HCl (pH 6.8); 100 mM NaCl; 5 mM dithiothreitol; 0.2 mM EDTA; 0.4 mM $CaCl_2$; 0.1 mM phenylmethylsulfonyl fluoride, at a protein concentration of approx. 5 mg ml^{-1}.

For the detergent extraction and mixed-micelle gel chromatography the procedures described in [12] were used with minor modifications. The membranes were partially solubilized with Triton X-100 at a (w/w) detergent/protein ratio of 2. The supernatant from 30 min centrifugation at 200 000 × *g* was applied to Sepharose CL 6B column equilibrated with a sonicated suspension of asolectin and Tween-20 in the same buffer medium as was used for the solubilization. Upon elution with the same lipid–detergent mixture, the (Ca^{2+} + Mg^{2+})-ATPase activity was eluted from the column in the front fractions of the protein peak. The fractions with highest activity were pooled, treated in batches for 1 h with 0.5 g of activated Biobeads SM2 to remove excess detergent, and subsequently concentrated ten times on an Amicon XM-50 filter.

The final protein concentration was 1.5 mg ml^{-1}. The samples were assayed immediately after preparation, unless otherwise indicated. When frozen in liquid nitrogen and then stored at −70°C the samples showed no loss of (Ca^{2+} + Mg^{2+})-ATPase activity in the absence of calmodulin. The calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase, however, was approx. 30% lower than for fresh samples.

Marker enzymes were assayed as described in the literature: (Na^+ + K^+)-ATPase (EC 3.6.1.3) in [13]; alkaline phosphatase (EC 3.1.3.1) in [14]; rotenone-insensitive NADH cytochrome *c* reductase (EC 1.6.99.3) in [15] and cytochrome *c* oxidase (EC 1.9.3.1) in [16]. For the assay of (Ca^{2+} + Mg^{2+})-ATPase, Ca^{2+}-

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

buffered solutions containing 1 mM EGTA were used. Assay method and calculation of the free calcium concentration were as in [6]. Calmodulin was purified from bovine brain [17]; the concentration was 10 μg/ml unless otherwise indicated. Protein was determined using a modified Lowry et al. method [18] with bovine serum albumin as a standard.

Materials were: asolectin was from Paesel GmbH and Co. (Frankfurt, FRG); Triton X-100 was from Merck; Tween-20 was from Supelco Inc.

3. Results and discussion

The crude membrane preparation used as a starting material for the purification of $(Ca^{2+} + Mg^{2+})$-ATPase was assayed for different marker enzymes.

The enrichment factor e.f. (mean ± S.E.M., $n = 12$) was defined as the ratio of the activity in the crude membrane preparation versus the activity in the homogenate.

The preparations were preferentially enriched in basal–lateral membranes as indicated by $(Na^+ + K^+)$-ATPase: e.f. = 5 ± 0.38. Mitochondria had been partially removed as indicated by cytochrome *c* oxidase: e.f. = 0.6 ± 0.15. Brush border membranes (alkaline phosphatase: e.f. = 2.3 ± 0.16) and internal membranes (NADH cytochrome *c* reductase: e.f. = 1.91 ± 0.18) were present in considerable amounts.

The preparation contained an ATP-dependent Ca^{2+} pump. About 30 nmol Ca^{2+} per mg protein were accumulated in 30 min at 37°C from a solution buffer with 10 μM free Ca^{2+}. This is similar to other transport data for renal membrane preparations [1,2]. The concomitant $(Ca^{2+} + Mg^{2+})$-ATPase in these conditions, which are optimal for Ca^{2+} uptake, was however not detectable. This is essentially due to the high background of Mg^{2+}-ATPase in the preparation. The $(Ca^{2+} + Mg^{2+})$-ATPase activity became apparent after the purification procedure described in the experimental section. The basal Mg^{2+}-ATPase activity of the purified preparation and the stimulation by Ca^{2+} and by Ca^{2+} and calmodulin are summarized in table I.

Table 1
ATPase components in renal membrane fractions after detergent solubilization and mixed micelle chromatography

	ATPase activity
Basal Mg^{2+}-dependent ATPase (10^{-4} M of ouabain)	45.8 ± 1.8 (7)
$(Ca^{2+} + Mg^{2+})$-dependent ATPase (10^{-5} M of free Ca^{2+})	26.6 ± 1.4 (8)
$(Ca^{2+} + Mg^{2+})$-dependent ATPase (10^{-5} M of free Ca^{2+} + calmodulin 10 μg ml^{-1})	70.3 ± 2.7 (5)

The values are means ± S.E.M. expressed as nmol P_i per mg protein per min. The number of observations is given in parentheses. The activities were determined at 37°C by the procedure described in [6]

The activation of the enzyme by Ca^{2+}, in the presence and in the absence of calmodulin is illustrated in fig.1. The $(Ca^{2+} + Mg^{2+})$-ATPase in our preparation had a high affinity for free Ca^{2+}. A concentration of 5 μM of free Ca^{2+} gave a half-maximal stimulation of the ATPase activity. In the presence of 10 μg ml^{-1} of calmodulin, the concentration of free Ca^{2+} needed for half-maximal activation was lowered to 1.5 μM. Concentrations of free Ca^{2+} above 10 μM were inhibitory both in the absence and in the presence of calmodulin. The stimulatory effect of calmodulin on the $(Ca^{2+} + Mg^{2+})$-ATPase is further illustrated in fig.2. A half-maximal activation was observed at a calmodulin concentration of 85 ng ml^{-1} (5 nM). The maximal activity observed in the presence of calmodulin was 70 nmol P_i per mg protein and per minute, which is 275% of the activity in the absence of calmodulin.

The high affinity $(Ca^{2+} + Mg^{2+})$-ATPase observed in this study can be considered as the enzymatic basis

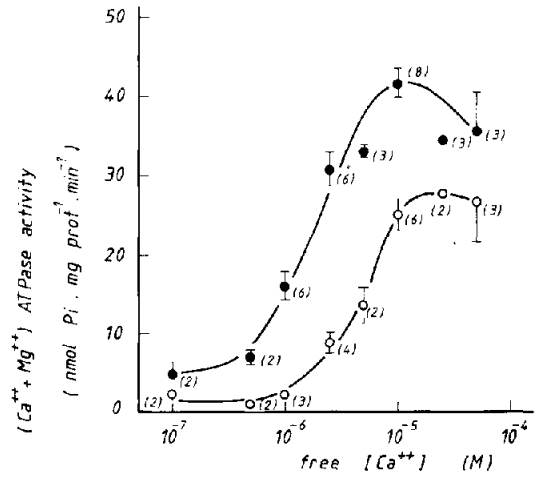

Fig.1. Calcium dependence of the renal high affinity $(Ca^{2+} + Mg^{2+})$-ATPase, in the presence (•) and in the absence (○) of calmodulin (10 μg ml^{-1}). Assay conditions are as indicated under Experimental except that the preparation was frozen in liquid nitrogen and stored at −70°C before use. The error bars represent ± S.E.M.; the number of observations is indicated in parentheses.

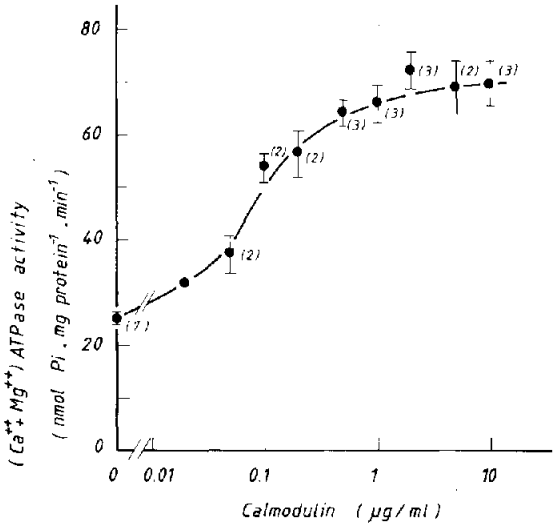

Fig.2. Dependence of the renal high affinity (Ca^{2+} + Mg^{2+})-ATPase activity on calmodulin concentration. The free calcium concentration in this experiment was 10 μM. Assay conditions were as described under Experimental. The error bars represent ± S.E.M.; the number of observations is indicated in parentheses.

for the ATP-driven Ca^{2+} uptake in renal membrane preparations. The essential characteristic which supports this statement is the affinity for free Ca^{2+} in the micromolar range. This is also typical for (Ca^{2+} + Mg^{2+})-ATPase from those preparations where its transport function has been demonstrated (sarcoplasmic reticulum [19] and erythrocytes [20]). A further characteristic of the renal (Ca^{2+} + Mg^{2+})-ATPase is its activation by calmodulin, which is well documented for the (Ca^{2+} + Mg^{2+})-ATPase from erythrocytes [21]. Calmodulin stimulation was also found for ATP-dependent Ca^{2+}-transport across membranes from adipocytes [22]. In that case evidence was presented for a plasma membrane localization of calmodulin-sensitive transport. From our study, no statement can be made about the subcellular localization of the (Ca^{2+} + Mg^{2+})-ATPase, since both plasma membranes and internal membranes are enriched in our initial crude membrane preparation. It is possible that the (Ca^{2+} + Mg^{2+})-ATPase observed here originates from both types of membranes. In that case it remains to be clarified whether calmodulin affects both in the same way. Calmodulin has been purified from porcine renal medulla [23] and its effects on renal adenylate cyclase were described. The possibility that calmodulin may have a role in renal transport phenomena is supported by our observations.

Acknowledgements

We gratefully acknowledge the skillful assistance of Miss L. Bauwens throughout the experiments. This work was supported by grant 3.0087.74 of the FWGO.

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