Localization and properties of a high-affinity (Ca^{2+} + Mg^{2+})-ATPase in isolated kidney cortex plasma membranes

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1. INTRODUCTION

Two different calcium-transport systems have been found to operate in parallel in kidney basal–lateral plasma membranes: an ATP-dependent Ca^{2+}-transport and a Na^+/Ca^{2+} exchange [1]. It was suggested that a high-affinity Ca^{2+}-ATPase was involved in the ATP-dependent Ca^{2+} uptake by inverted basal–lateral membrane vesicles, and thus in the extrusion of Ca^{2+} from the tubular cell in vivo. However, very little is known about the enzyme itself. Kinne Safran and Kinne [2] found a low-affinity Ca^{2+}-ATPase activity in kidney basal–lateral membranes. De Smedt et al. [3] reported a partial purification of a high-affinity, calmodulin-dependent (Ca^{2+} + Mg^{2+})-ATPase from kidney cortex microsomes, but the origin of the enzyme was not established in [3]. Van Os et al. [4] reported in an abstract the presence of a Ca^{2+}-stimulated ATPase with high affinity for calcium ($K_{m_{Ca^{2+}}}$ 0.4 μM) in a basal–lateral plasma membrane fraction from the kidney. Here, we report the localization, kinetics, calmodulin dependence and inhibitor sensitivity of a high-affinity (Ca^{2+} + Mg^{2+})-ATPase in highly purified basal–lateral plasma membranes from rat kidney cortex.

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid; HEDTA, *N*-(2-hydroxyethyl)ethylenediamine-*N,N'*,N'-triacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

2. METHODS

Basal–lateral plasma membranes were isolated from slices of outer kidney cortex of rats by a modification of the Percoll gradient centrifugation procedure in [5]. The isolation medium contained: 250 mM sucrose, 10 mM triethanolamine–HCl, 0.5 mM EGTA and 0.1 mM phenylmethyl-sulfonylfluoride (pH 7.6). A crude plasma membrane fraction was isolated by differential centrifugation as in [5]. The membranes were suspended in exactly 30 ml isolation medium, homogenized lightly by hand and mixed with 4.0 ml standard isotonic Percoll solution (SIP), made up of 9 vol. stock Percoll and 1 volume of 2.5 M sucrose. The density of SIP was 1.149 g/cm^3, and final Percoll concentration in the gradients was 10.4%. The membranes were centrifuged at 36 900 × $g_{av.}$ in a Sorvall RC-2B centrifuge with SS-34 fixed angle rotor and an integrator set to 8.00 × 10^9 ω^2/dt (run time of ~35 min). The gradient was unloaded by pumping 50% sucrose to the bottom of the tube and collecting 2 ml fractions from the top. To collect the basal–lateral membrane fraction the initial 3 ml were discarded and the following 7 ml were pooled. The collected fraction was diluted to 30 ml with 160 mM KCl, 20 mM Hepes (pH 7.4) and centrifuged for 40 min at 218 000 × g_{av} in a Sorvall OTD-75B ultracentrifuge with T-864 rotor. The membranes which sedimented on top of a glass–Percoll pellet were suspended with a syringe in ~1 ml of the same KCl-Hepes solution. With the above improvements, the method proved to be excellently reproducible and yielded a highly purified basal–lateral membrane fraction.

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To deplete the membranes of endogenous calmodulin, the pellet from the 218 000 × g_{av} centrifugation was suspended and homogenized in 15 ml 20 mM Hepes, 4 mM EGTA solution (pH 7.4). The suspension was left on ice for 30 min, then 15 ml 1.2 M KCl, 20 mM Hepes (pH 7.4) was added and the membranes were centrifuged at 218 000 × g_{av} for 40 min. The pellet was suspended in about 1 ml of 160 mM KCl, 20 mM Hepes (pH 7.4). The final membrane suspension had 8–12 mg protein/ml.

(Ca^{2+} + Mg^{2+})-ATPase activity was measured by a coupled enzyme assay as in [6]. The method was adapted to the LKB 2086 enzyme kinetics analyzer. The incubation medium contained: 160 mM KCl, 20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM HEDTA, 2.5 mM ouabain, 0.4–0.9 mM $CaCl_2$ and 10–30 μg membrane protein/ml. Free [Ca^{2+}] in the mixture were calculated as described in Appendix 1. The reaction was started by automatic injection of 0.1 ml enzyme–substrate mixture into 1.05 ml incubation medium at 37°C. The mixture contained: 5.75 mM ATP, 5.75 mM phosphoenolpyruvate, 2.7 mM NADH, 11.5 IU pyruvate kinase/ml and 11.5 IU/ml lactate dehydrogenase, dissolved in the incubation medium and adjusted to pH 7.4. The reaction rate was measured for 1 min after an initial delay of 20 s. Each measurement was made in quintuplicate to obtain the precision necessary in face of a high background Mg^{2+}-ATPase activity.

(Na^+,K^+)-ATPase and leucylaminopeptidase activities were measured with the LKB 2086 enzyme analyzer as in [7].

3. RESULTS

The distribution of the marker enzymes: (Na^+, K^+)-ATPase for the basal–lateral membranes and leucylaminopeptidase for the brush border membranes on a Percoll gradient is shown in fig.1. (Na^+,K^+)-ATPase separated as a sharp peak at ~ 1.04 g/cm^3, as determined with the density marker beads run on a parallel gradient. The cross-contamination with the brush border membranes was much smaller than in the free flow electrophoresis, which was used for the isolation of basal–lateral membranes in [1]. The distribution of (Ca^{2+} + Mg^{2+})-ATPase correlated very closely with the distribution of (Na^+,K^+)-ATPase. Practically no (Ca^{2+} + Mg^{2+})-ATPase activity was detected in the brush border membrane fractions. These results indicate an exclusive localization of (Ca^{2+} + Mg^{2+})-ATPase in the basal–lateral membranes of renal tubular cells. In table 1 the enzyme activities in pooled basal–lateral membrane fractions are shown. A high specific activity and an enrichment factor of 32 for (Na^+,K^+)-ATPase indicate a rather high purity of this fraction. The (Ca^{2+} + Mg^{2+})-ATPase activity is rather small as compared to both the (Na^+,K^+)-ATPase and the Mg^{2+}-ATPase activities, and could only be measured by multiple automated analysis.

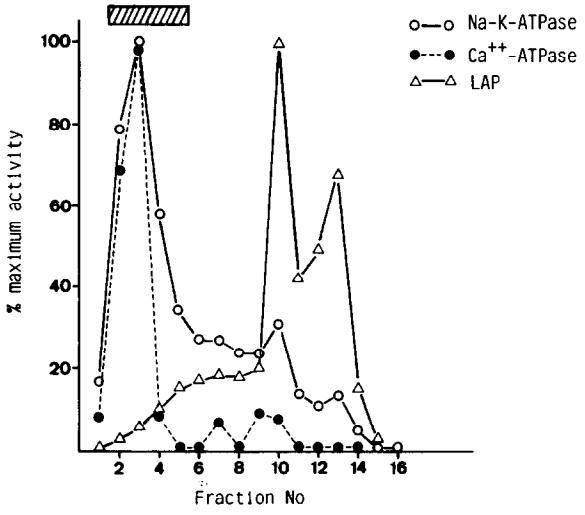

Fig.1. Distribution of (Ca^{2+} + Mg^{2+})-ATPase and plasma membrane marker enzymes on the Percoll gradient. (Ca^{2+} + Mg^{2+})-ATPase activity was measured as in section 2 in the presence of 3.3 μM free Ca^{2+}; the hatched bar indicates the fractions which were pooled.

In fig.2, the calcium concentration dependence of the Mg^{2+}-ATPase activity in the basal–lateral plasma membranes is shown. A stimulation of the ATPase activity was already seen at sub-μM free Ca^{2+} levels. Calmodulin (bovine brain, Fluka) had no effect on the Ca^{2+}-stimulated ATPase activity in this particular preparation, although in some preparations a variable amount of stimulation was observed. This probably reflected a variable degree of calmodulin extraction from the membranes during isolation in an EGTA-containing medium. The Ca^{2+} stimulation of the ATPase activity was nearly completely abolished by the calmodulin inhibitor R-24571 [8]. The basal Mg^{2+}-ATPase activity was

Table 1

Marker enzyme activities in the isolated kidney basal–lateral plasma membranes

	Homogenate	Basal–lateral membranes	Enrichment
(Na^+,K^+)-ATPase	47 ± 10	1491 ± 157	32
Leucylaminopeptidase	124 ± 15	135 ± 16	1.1
Mg^{2+}-ATPase	–	680 ± 65	–
$(Ca^{2+} + Mg^{2+})$-ATPase	–	80 ± 17	–

The enzyme activities are expressed as nmol mg protein^{-1} . min^{-1}, measured at 37°C. The values are means ± SEM from 6–14 expt

also considerably inhibited by R-24571. Although the results on inhibition by the so-called anti-calmodulin drugs must be interpreted with caution (see [9]), this result suggested a calmodulin dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase in the basal–lateral membranes.

In fig.3, the calmodulin dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase in calmodulin-depleted basal–lateral plasma membranes is shown. Calmodulin depletion by the hypotonic EGTA treatment resulted in a considerable decrease of the Ca^{2+} affinity of the enzyme. The addition of exogenous bovine brain calmodulin restored the high calcium sensitivity of the ATPase. In agreement with [4,10], a 'roll-over' phenomenon was observed; i.e., the Ca^{2+} stimulation of the ATPase was less than maximal at higher calcium concentrations.

The kinetic properties of the $(Ca^{2+} + Mg^{2+})$-ATPase in both native and calmodulin-depleted basal–lateral plasma membranes are summarized in table 2. The $K_{m_{Ca^{2+}}}$ 0.68 μM of the ATPase in native membranes is in a reasonable agreement with the $K_{m_{Ca^{2+}}}$ 0.5 μM of the ATP-dependent Ca^{2+} uptake [1]. In calmodulin-depleted membranes the $K_{m_{Ca^{2+}}}$ was approximately doubled as compared to the native membranes. The $K_{m_{Ca^{2+}}}$ decreased to 0.24 μM upon addition of exogenous calmodulin. The latter value is well below the 0.45 μM free Ca^{2+} level measured in kidney cells [11]. Thus, the properties of the enzyme are compatible

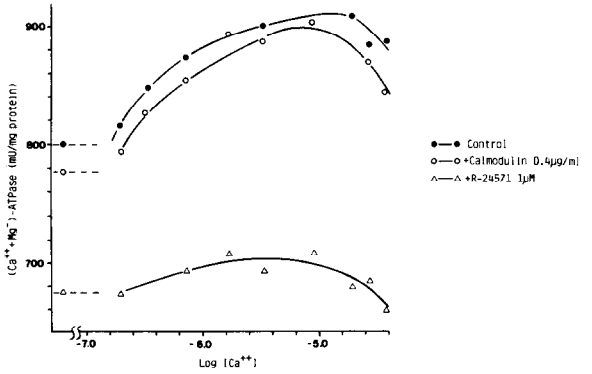

Fig.2. Ca^{2+}-stimulation of Mg^{2+}-ATPase in isolated basal–lateral plasma membranes: 1 mU represents 1 nmol ATP split/min at 37°C; (●——●) control; (○——○) + 0.4 μg calmodulin/ml; (△——△) + 1 μM R-24571.

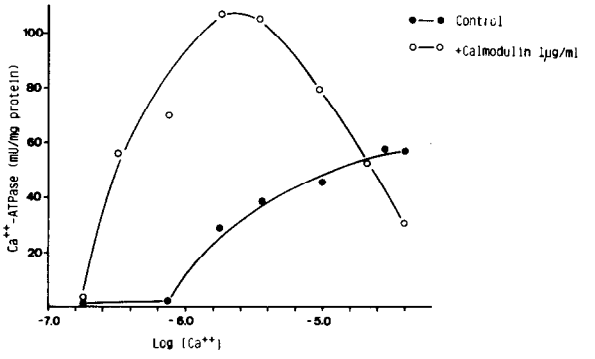

Fig.3. Ca^{2+} concentration dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase activity in basal–lateral plasma membranes depleted of endogenous calmodulin by hypotonic EGTA extraction: (●——●) depleted membranes; (○——○) depleted membranes + 1 μg calmodulin/ml.

Table 2

Kinetic parameters of the $(Ca^{2+} + Mg^{2+})$-ATPase in basal–lateral plasma membranes of rat kidney cortex

	$K_{m_{Ca^{2+}}}$ (μM)	V_{max} (nmol . mg^{-1} . min^{-1})
Native membranes	0.68 ± 0.14	80 ± 17
Membranes extracted with hypotonic EGTA:		
– calmodulin	1.17 ± 0.18	75 ± 11
+ calmodulin 1 μg/ml	0.24 ± 0.05	105 ± 12

Values are means ± SEM from 5–8 expt

with its role as a calcium-transporting system.

In fig.4, the inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase by vanadate is shown. The enzyme is highly sensitive to vanadate, with half-maximal inhibition at 0.8 μM. The $(Ca^{2+} + Mg^{2+})$-ATPase in kidney basal–lateral membranes is also similar to other plasma membrane Ca^{2+}-ATPases and different from sarcoplasmic reticulum Ca^{2+}-ATPase [6,12].

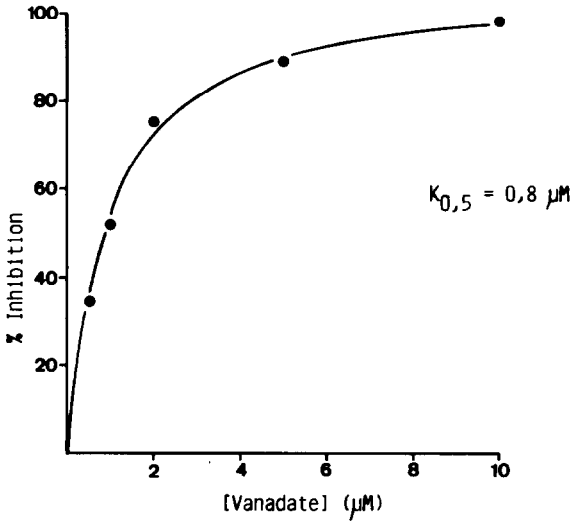

Fig.4. Inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase in the basal–lateral membranes by sodium orthovanadate. $(Ca^{2+} + Mg^{2+})$-ATPase activity was measured in the presence of 3.3 μM free Ca^{2+}. Control activity was 79 mU/mg protein.

4. DISCUSSION

These data provide evidence that the basal–lateral plasma membranes of renal tubular cells contain a high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase activity, whereas no such activity is observed in the brush border membranes. The origin of the enzyme is ascertained by its distribution on a Percoll gradient in parallel to the (Na^+,K^+)-ATPase, as well as its high sensitivity to vanadate, which seems to be characteristic of the plasma membrane Ca^{2+}-ATPases [12]. The localization and the $K_{m_{Ca^{2+}}}$ of the enzyme are practically the same as the localization and the $K_{m_{Ca^{2+}}}$ of the ATP-dependent Ca^{2+} uptake by kidney plasma membranes isolated by free-flow electrophoresis [1]. This suggests that the ATP-dependent Ca^{2+} uptake by the kidney basal–lateral plasma membrane vesicles is an expression of the $(Ca^{2+} + Mg^{2+})$-ATPase activity. The enzyme appears to be calmodulin-sensitive, as indicated by its inhibition by the calmodulin inhibitor R-24571, and by the restoration of the high calcium affinity of the ATPase in calmodulin-depleted membranes by exogenously added calmodulin. The $K_{m_{Ca^{2+}}}$ 0.68 μM in the native membranes and 0.24 μM in the presence of exogenous calmodulin are in a reasonable agreement with the $K_{m_{Ca^{2+}}}$ 0.4 μM reported in [4], if it is taken into account that the membranes can become partially depleted of endogenous calmodulin during the isolation procedure. These $K_{m_{Ca^{2+}}}$-values are considerably lower than $K_{0.5_{Ca^{2+}}}$ 1.5 μM for the purified enzyme in the presence of calmodulin [3]. The reasons of this discrepancy are not clear. It cannot be excluded that

the calmodulin sensitivity is partially lost during enzyme isolation. Alternatively, different methods of calculation of free Ca^{2+} concentrations might have resulted in divergent numerical results.

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APPENDIX

Since the measurements of sub-μM free Ca^{2+} levels by the Ca^{2+}-sensitive electrode were not feasible in the presence of Mg^{2+} in the medium, they were calculated by a computerized iterative method for calculation of the concentrations of species present in mixtures of associating ions, as in [13]. The following general equations were used:

$$A_i = a_i + \sum_{j=1}^{m} (\alpha_{ij} K_j \prod_{k=1}^{n} a_k^{\alpha}) \quad (1)$$

where

A_i = total concentration of ion i
a_i = free concentration of ion i
α_{ij} = number of molecules of i in complex j
K_j = association constant of complex j

Equation (1) is rearranged to express any of the free concentrations a_i in terms of the total concentrations A_i [13]:

$$a_i = A_i a_i / [a_i + \sum_{j=1}^{m} (\alpha_{ij} K_j \prod_{k=1}^{n} a_k^{\alpha})] \quad (2)$$

Equations (2) for each species present in the mixture were programmed into a Hewlett-Packard HP-9815 calculator. The results converged to a self-consistent solution after 30–35 iterations. The following stability constants [14,15] corrected for ionic strength = 0.15 and temp. = 37°C were used:

EGTA
$pK_1 = 9.35$, $pK_2 = 8.73$, $pK_3 = 2.78$, $pK_4 = 2.12$,
$\log K_{Ca\text{-}EGTA} = 10.64$, $\log K_{Ca\text{-}H\text{-}EGTA} = 5.33$,
$\log K_{Mg\text{-}EGTA} = 5.41$, $\log K_{Mg\text{-}H\text{-}EGTA} = 1.43$

HEDTA
$pK_1 = 9.74$, $pK_2 = 5.40$, $pK_3 = 2.75$
$\log K_{Ca\text{-}HEDTA} = 8.02$, $\log K_{Ca\text{-}H\text{-}HEDTA} = 1.38$
$\log K_{Mg\text{-}HEDTA} = 7.10$, $\log K_{Mg\text{-}H\text{-}HEDTA} = 1.43$

ATP
$pK_1 = 6.67$, $pK_2 = 4.03$, pK_3 – neglected
$\log K_{Ca\text{-}ATP} = 3.73$, $\log K_{Ca\text{-}H\text{-}ATP} = 1.8$
$\log K_{Mg\text{-}ATP} = 4.14$, $\log K_{Mg\text{-}H\text{-}ATP} = 2.0$