The plasma membrane Ca^{2+} pump from proximal kidney tubules is exclusively localized and active in caveolae

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Abstract Plasma membrane Ca^{2+}-ATPase is involved in the fine-tuned regulation of intracellular Ca^{2+}. In this study, the presence of Ca^{2+}-ATPase in caveolae from kidney basolateral membranes was investigated. With the use of a discontinuous sucrose gradient, we show that Ca^{2+}-ATPase is exclusively located and fully active in caveolin-containing microdomains. Treatment with methyl-β-cyclodextrin – a cholesterol chelator – leads to a spreading of both caveolin and completely inactive Ca^{2+}-ATPase toward high-density fractions. These data support the view that Ca^{2+} fluxes mediated by Ca^{2+}-ATPase in kidney epithelial cells occur only in caveolae, being strictly dependent on the integrity of these microdomains.

Keywords: Caveolae; Ca^{2+}-ATPase; Kidney; Basolateral membranes; Ca^{2+} homeostasis; Calmodulin

1. Introduction

The main parts of the regulatory system involved in cell calcium homeostasis are the different sets of transport that are present either in the membranes of internal stores as sarco(endo)plasmic reticulum, which is driven through different sarco(endo)plasmic reticulum Ca^{2+}-ATPases [1], or in the plasma membrane, where it is driven through the Na^+–Ca^{2+} exchanger and the plasma membrane Ca^{2+}-ATPase [2–7]. Since it was first reported by Yamada [8], rapid growth in caveolae research has brought major advances in understanding its structure, as well as the development of methods for purifying and characterizing its composition. These studies were carried out in many different cell types, where these plasma membrane microdomains were detected [9–11]. However, there are some reports that argue whether caveolae are really involved in the functions attributed to them (for example see [12]).

Quantitative analyses by immunofluorescence and immunocitochemistry of endothelial cells, smooth muscle cells, cardiac muscle cells, epidermal keratinocytes, mesothelial cells and capillary endothelial cells have shown that Ca^{2+}-ATPase was found to be concentrated 18–25-fold in the caveolar membrane compared with the non-caveolar portion of the plasma membrane [13,14]. However, there are no biochemical data reporting the activity of Ca^{2+}-ATPase in its caveolar localization. The caveolar localization of the Ca^{2+} pump appears to be crucial for kidney function, even though the first studies on caveolae have demonstrated that these microdomains are not abundant in kidney tubules [13]. It was shown that the knock out of the caveolin-1 gene promotes the progressive steps required for urinary calcium lithiasis [15]. Moreover, it was observed that the dysfunction of caveolin- and cholesterol-rich microdomains is directly correlated and potentially involved in the induction and maintenance phases of ischaemic and toxic forms of experimental renal failure [16], emphasizing the great importance of these specialized plasma membrane microdomains in renal physiology and pathology. These observations led us to investigate whether the Ca^{2+}-ATPase resident in the basolateral membranes (BLM) of proximal tubule cells is active in its caveolar localization and also to compare the Ca^{2+}-ATPase activity from caveolar and non-caveolar fractions in the above kidney segment. In this tubule segment more than 70% of the glomerular filtrate is recovered for the body liquid compartments, and reabsorption of fluid appears to be highly sensitive to the cytosolic Ca^{2+} fluctuations [17] which are controlled in part by the BLM Ca^{2+} pump [18].

2. Materials and methods

2.1. Materials

All the reagents used for isolation of the membranes, sucrose gradient, Ca^{2+}-ATPase activity and protein determination as well as bovine brain calmodulin, were from Sigma Chemical Co. (St. Louis, MO). The reagents used for SDS–PAGE and Western blotting (ECL™ and Hyperfilm™) were from Amersham (Buckinghamshire, UK). The monocolonal antibody anti-PMCA (5F10) and the polyclonal antibody anti-caveolin-1 (anti-cav-1) were from Affinity BioReagents (Golden, CO) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively.

2.2. Isolation of BLM

BLMs from pig kidney proximal tubule cells were isolated and purified using the Percoll gradient method [19] and stored in 250 mM sucrose under liquid N_2 at a final protein concentration of 30–35 mg/ml. Under these conditions, Ca^{2+}-ATPase activity is preserved for at

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Abbreviations: BLM, basolateral membranes; MβCD, methyl-β-cyclodextrin; Polidocanol, polyoxyethylene 9 lauryl ether

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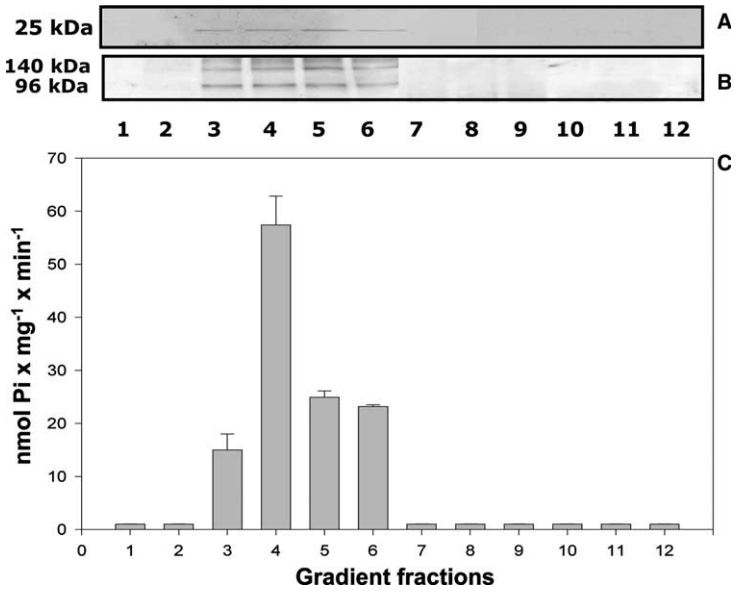

Fig. 1. Association between caveolin and full active Ca^{2+}-ATPase activity in plasma membranes from kidney tubule cells. Representative Western blot of caveolin (25 kDa) polyclonal antibody anti-cav-1 (1:1000) in membrane fractions separated in a polidocanol-containing discontinuous sucrose gradient after centrifugation for 16 h [repeated five times with different preparations] (A). Representative Western blot of Ca^{2+}-ATPase using monoclonal antibody anti-PMCA, 5F10 clone (1:1000), in the same gradient (B). Ca^{2+}-ATPase activity in the fractions indicated on the abscissa (C). The Ca^{2+}-ATPase activity control value from different BLM preparations, incubated at 4 °C without centrifugation for 16 h, was 10 ± 2 nmol $P_i \times mg^{-1} \times min^{-1}$ ($n = 5$).

least 3 months. This preparation contains 30–40% of unsealed membrane fragments [19]. Protein determination was carried out by the Folin-phenol method [20], using bovine albumin as standard. The specific activity of the BLM marker (Na^+–K^+)ATPase was ~15-fold enriched when compared to the initial kidney cortex-corticis homogenate. Different preparations selected at random showed that residual contamination with subcellular membrane markers was minimal [21].

2.3. Caveolae fractionation

BLM-enriched fractions obtained as described above were treated with polidocanol (1%, v/v). This material was sonicated three times (10 s each) and then fractionated in a discontinuous sucrose gradient (5–45%), as previously described [22]. The 12 top to bottom fractions obtained (1 ml each) were used for Western blotting assays and determination of Ca^{2+}-ATPase activity.

2.4. Ca^{2+}-ATPase activity determination

Ca^{2+}-ATPase activity was measured using the colorimetric method described by Taussky and Shorr [23]. The native BLM fraction or the different sucrose gradient fractions were incubated in the following reaction medium (1 ml): 50 mM bis-Tris propane–HCl (pH 7.4), 10 mM NaN_3, 1 mM ouabain, 120 mM KCl, 5 mM $MgCl_2$, 0.2 mM EGTA and 0.27 mM $CaCl_2$ (20 μM free Ca^{2+}). The reaction was started by adding 5 mM ATP to the reaction medium. After 20 min at 37 °C the reaction was stopped with two volumes of a cold activated charcoal suspension in 0.1 N HCl. An aliquot of the supernatant obtained after centrifugation ($600 \times g$, 40 min) was used for the colorimetric assay. Spontaneous P_i release was measured in tubes run in parallel in which the membranes were added after the charcoal. The Ca^{2+}-ATPase activity was measured by the difference of the ATP hydrolysis in the presence and absence (0.2 mM EGTA) of $CaCl_2$. Free Ca^{2+} concentration was calculated using a computer program that took into account the different species involved in the equilibrium between EGTA, Ca^{2+}, ATP, Mg^{2+}, H^+ and K^+, and the influence of ionic strength on the association constant for the Ca–EGTA complex [24,25].

2.5. Preparation of calmodulin-depleted membranes

Calmodulin-depleted BLM were obtained after incubation of the membranes in mild alkaline (pH 7.8) hypotonic (10 mM Tris–HCl, 2 mM EDTA) buffer according to Niggli et al. [26] with slight modifications [27].

2.6. Preparation of cholesterol–cyclodextrin inclusion complex

The inclusion complex of cholesterol and MβCD was prepared according to Klein et al. [28] with slight modifications. Briefly a solution of MβCD in water (0.2 M) was supplied with cholesterol under continuous stirring at room temperature. When the dextrin was fully loaded with cholesterol (cholesterol–MβCD molar ratio 1.8:1), an aliquot of the clear solution containing the complex was added to the Ca^{2+}-ATPase assay to give a final concentration of 75 mg/ml.

2.7. Electrophoresis and Western blotting

After sodium dodecylsulfate–polyacrylamide gel electrophoresis (15% acrylamide) of the BLM [29], proteins were transferred to a nitrocellulose membrane and incubated with the desired antibodies (see legend to the figures for the dilutions). After four washes in TBS-T (5 min each), the membranes were incubated for 1 h at room temperature with an anti-rabbit peroxidase conjugate antibody (dilution 1:5000 in TBS-T solution). The membranes were washed again as described above, and the bands were visualized using the ECL system kit.

3. Results and discussion

3.1. Localization of the Ca^{2+} pump from BLM in caveolin-rich fractions

With the use of a policlonal antibody against caveolin-1, we detected a clear signal of abundant caveolin – and therefore caveolae – in BLM derived from kidney proximal tubule cells. Fig. 1A shows that caveolin is only found in the low density, cholesterol-rich fractions of the sucrose gradient (fractions 3–6) [9,30]. These fractions are the same as those in which Ca^{2+}-ATPase was detected by the monoclonal antibody anti-plasma membrane Ca^{2+}-ATPase (5f10), as shown in Fig. 1B (fractions 3–6). In addition, the Ca^{2+}-ATPase activity is found exclusively in the same fractions in which caveolin-1 and plasma membrane Ca^{2+}-ATPase are detected by the corresponding antibodies (Fig. 1C). These data clearly show that Ca^{2+}-ATPase is compartmentalized and functional only in caveolae.

3.2. Caveolae disruption inactivates plasma membrane Ca^{2+}-ATPase

To clarify whether Ca^{2+}-ATPase really depends on the integrity of caveolae to work, we carried out experiments where the BLMs were pre-incubated, prior to fractionation in the sucrose gradient, with MβCD, a well-known caveolae disrupter due to its action in sequestering cholesterol. Many studies have shown that cholesterol is a molecule critically involved in caveolae stability and function [11,31,32] since this membrane microdomain is highly sensitive to cholesterol depletion [22,31]. Fig. 2A clearly shows that pre-treatment of the BLM with MβCD promotes disruption of caveolae as judged by the spread of caveolin detection along the entire gradient, with a marked increase in the high-density regions (fractions 10–12). It has been shown that after cholesterol depletion by MβCD, the caveolin-containing complexes lose their low-density property [31,33]. Therefore, it is expected that, in the presence of MβCD, complexes of caveolin with cytoskeleton proteins should also be present in the high-density regions of the gradient [33] as shown in Fig. 2A. Plasma membrane Ca^{2+}-ATPase was also distributed along the entire gradient as revealed by its broader detection in the Western blotting (Fig. 2B). When these fractions were assayed for Ca^{2+}-ATPase activity, we were not able to detect any functional pool of Ca^{2+}-ATPase (main panel in Fig. 2C), thus suggesting that the caveolae microenvironment is strictly necessary for Ca^{2+}-ATPase function in the BLMs of kidney cells. Cholesterol depletion by MβCD also critically impairs Ca^{2+}-ATPase activity when native membrane preparations are used, instead of the solubilized preparation employed in the gradient shown in the main panel of Fig. 2C. The inset to Fig. 2C shows the concentration-dependent inhibition by MβCD of the Ca^{2+}-ATPase activity of intact BLM. It is of interest to mention that the $(Na^+ + K^+)$ATPase of these membranes is also abolished by 50 mM MβCD (data not shown).

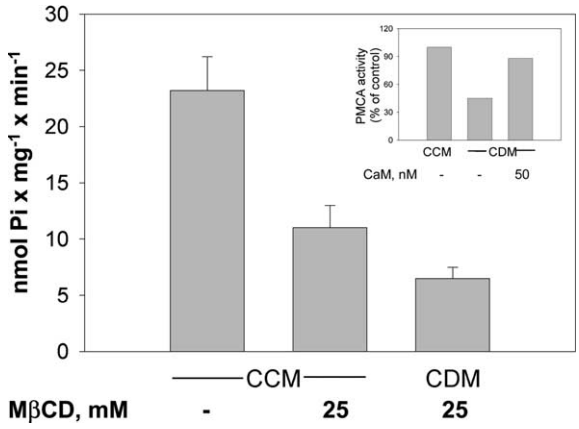

Fig. 3. Influence of MβCD on Ca^{2+}-ATPase from calmodulin-depleted membranes. *Main panel:* Ca^{2+}-ATPase activity was measured in the conditions shown on the abscissa. CCM: calmodulin-containing membranes; AHT: alkaline hypotonic treatement (see Section 2). *Inset:* Ca^{2+}-ATPase activity measured in calmodulin-containing membranes (CCM), in calmodulin-depleted membranes (CDM), and in membranes first depleted from their endogenous calmodulin by alkaline hypotonic treatment and then supplied with 50 nM bovine brain calmodulin.

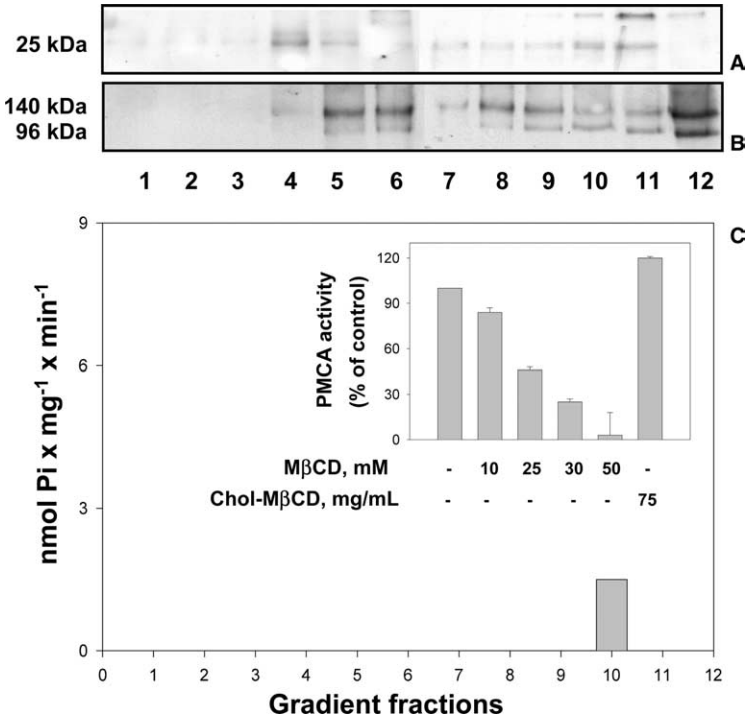

Fig. 2. Correlation between disruption of caveolae and inactivation of Ca^{2+}-ATPase. Representative Western blot of caveolin in membrane fractions treated with 50 mM MβCD (A). Western blot of fractions using antibody against Ca^{2+}-ATPase. The specific dilutions of the anti-cav-1 and 5F10 antibodies were as in legend to Fig. 1(B). Ca^{2+}-ATPase activity in membranes treated with MβCD (C); *main panel*, fractions of polidocanol-containing sucrose gradient (with 50 mM MβCD); *inset*, native membranes assayed for Ca^{2+}-ATPase activity in the presence of different MβCD concentrations or 75 mg/ml of cholesterol–MβCD complex (1.8:1 molar ratio), as shown on the abscissa. Results are presented as means ± SEM of five different experiments carried out in triplicate.

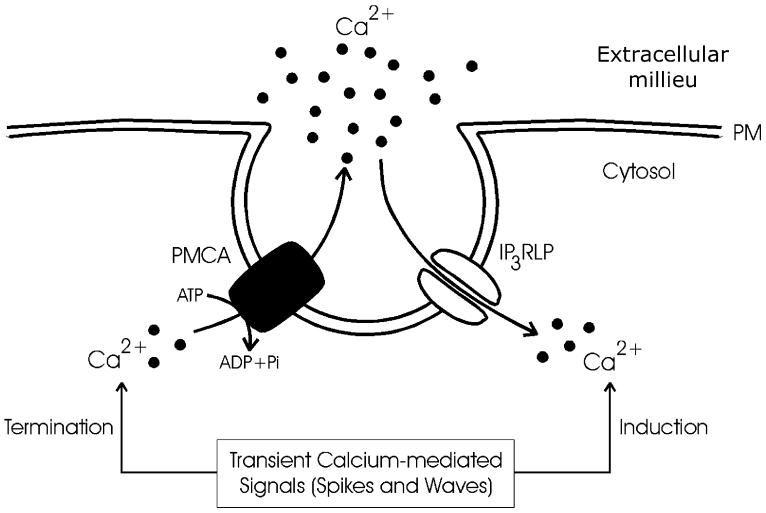

Fig. 4. Model for the fine-tuned regulation of cellular Ca^{2+} mediated by caveolar Ca^{2+}-ATPase after Ca^{2+} entry through the IP_3 receptor-like protein (IP_3RLP) (see also [43]). The pathways for passive Ca^{2+} entry and active Ca^{2+} efflux localized in close vicinity in caveolar microdomains would warrant the fine-tuned control of the intensity and duration of Ca^{2+} spikes and Ca^{2+} waves [43].

When an inclusion complex of cholesterol with MβCD [28,34] instead of free MβCD was used, there is no inhibition of Ca^{2+}-ATPase activity. This indicates that cholesterol extraction from the membranes – and not other non-specific effects – leads to loss of Ca^{2+}-ATPase activity, as is also the case for membrane receptors [28] and molecular signaling turnover [34]. However, when cholesterol was first removed by MβCD and caveolae were disrupted (Fig. 2A), incubation with the inclusion complex – which is believed to be a cholesterol donor [22,35] – is not enough to restore Ca^{2+}-ATPase activity. Thus, we propose that cholesterol has two effects on renal Ca^{2+}-ATPase. One would be to preserve the caveolar integrity needed for the stabilization and full activity of the pump. The second effect of cholesterol would be to contribute to the organization of an appropriate lipidic environment around the renal Ca^{2+}-ATPase molecules as it is the case for the $(Na^+ + K^+)$ATPase. Cholesterol stimulates the activity of the Na^+ pump in reconstituted systems [36,37] and this effect is not solely due to an increase in the hydrophobic thickness. Rather, specific interactions between $(Na^+ + K^+)$ATPase molecules leading to oligomerization and activation of the pump could be favored by cholesterol [37]. This could be also true for PMCA, which is also activated by oligomerization [38]. It should be mentioned that the basal Mg^{2+}-stimulated, Ca^{2+}-independent ATPase activity (2 mM EGTA) remains unchanged after MβCD treatment, indicating that disruption of caveolae by cholesterol removal specifically affects the Ca^{2+} pump.

3.3. Calmodulin depletion increases sensitivity to caveolar disruption

Ca^{2+}-ATPase is the only ion pump stimulated directly by calmodulin [2–7,26]. Thus, the next experiment addressed the question of whether calmodulin modifies inhibition of the pump by MβCD. Fig. 3 shows that inhibition is more pronounced when the membranes are depleted from their endogenous calmodulin by alkaline hypotonic treatment. This is an evidence that long-range intramolecular modifications of the domains embedded in the membrane moiety – which are induced by calmodulin binding at its cytosolic sites [6] – decrease the sensitivity of the pump toward cholesterol removal and caveolar disarrangements.

3.4. Conclusions

As pointed out above, Ca^{2+}-ATPase is the molecular machine involved in the fine-tuned regulation of cytosolic Ca^{2+} activity. Its caveolar localization could facilitate its interaction with modulators [39–41] and, especially, its cross-talk with the main channel structure associated with Ca^{2+} entry, the IP_3 receptor-like protein (IP_3RLP) [42,43]. Fig. 4 illustrates our proposal of a cross-talk between neighboring IP_3RLP and Ca^{2+}-ATPase, playing respective key roles in the induction and termination of Ca^{2+} signals in the cells. The high affinity of caveolin for cholesterol [44] would be responsible for caveolar oligomerization and stabilization, as well as for the proper packaging of Ca^{2+}-ATPase and IP_3RLP in the caveolar environment. Thus, the data presented here are in line with the view that alterations in cholesterol localization and caveolin expression in specific membrane microdomains, impair Ca^{2+} pumping activity and disrupt intracellular Ca^{2+} homeostasis and signaling. The Ca^{2+}-elicited events turned on by caveolar dysfunction might sustain activation of a myriad of cellular cascades and trigger several uncontrolled pathological processes [11,15,16,45].

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