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Acylphosphatase interferes with SERCA2a–PLN association

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Abstract

We previously reported that acylphosphatase, a cytosolic enzyme present in skeletal and heart muscle, actively hydrolyzes the phosphoenzyme (EP) of cardiac sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a), inducing an increased activity of this pump. We hypothesized that acylphosphatase-induced stimulation of SERCA2a, in addition to enhanced EP hydrolysis, may be due to a displacement of phospholamban (PLN), removing its inhibitory effect. To verify this hypothesis co-immunoprecipitation experiments were performed by adding recombinant muscle acylphosphatase to solubilized heart SR vesicles, used as a source of SERCA2a and PLN. With anti-acylphosphatase antibodies only SERCA2a was co-immunoprecipitated in an amount which increased in parallel to the concentrations of our enzyme. Conversely, using anti-SERCA2a antibody, both PLN and acylphosphatase were co-immunoprecipitated with SERCA2a, and the PLN amount in the precipitate decreased with increasing acylphosphatase concentrations. SERCA2a and PLN were co-immunoprecipitated by anti-phospholamban antibodies, but while the amount of precipitated phospholamban increased in the presence of acylphosphatase, the level of SERCA2a decreased. These preliminary results strengthen the supposed displacement of phospholamban by acylphosphatase.

Keywords: SERCA2a; Phospholamban; Acylphosphatase; Cardiomyopathy

Sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs) are 110-kDa transmembrane proteins that transport Ca^{2+} ions from the sarcoplasm to the lumen of the membrane system [1]. The energy for Ca^{2+} transport is provided by ATP; intermediate steps involve the formation and hydrolysis of a phosphoenzyme intermediate (EP) [2]. The cardiac sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) is regulated by its interactions with the intrinsic membrane protein phospholamban. Phospholamban (PLN) inhibits the activity of SERCA2a, but its phosphorylation by protein kinase A or Ca^{2+}-calmodulin kinase reverses the inhibitory interaction, presumably through dissociation of phosphorylated phospholamban from SERCA2a [3]. An additional regulatory effect on SERCA2a could be exerted by acylphosphatase (EC 3.6.1.7), a cytosolic 11 kDa enzyme well represented in cardiac muscle that catalyzes the hydrolysis of acylphosphates [4–6]. In previous studies we have shown that acylphosphatase actively hydrolyzes the EP intermediate of this transport system, an effect that results in an enhanced activity of the SR Ca^{2+} pump [7,8]. We have also demonstrated that acylphosphatase, in addition to its hydrolytic activity on EP, stimulated SERCA2a activity through another mechanism, probably due to its conformational properties. In fact, using an acylphosphatase mutant, Asn41Ser, devoid of catalytic activity but unchanged as for the three-dimensional structure [9], the stimulatory effect on SERCA2a was retained, but disappeared when the conformational structure of the mutant was altered by thermal denaturation. To explain these results we supposed that acylphosphatase could interact with SERCA2a taking the place of unphosphorylated PLN whose inhibitory effect could be removed [8]. In fact, NMR studies showed that acylphosphatase contains a structural motif where 12 residues (from 55 to 66) form an amphipathic α-helix with a prevalence of basic groups [10] that resembles that of the PLN cytoplasmic 1A domain, essential for the association with SERCA2a [11].

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In the present study, as a first approach to verify the supposed interaction, we performed a series of co-immunoprecipitation experiments. Mixtures of solubilized cardiac SR vesicles, used as a source of SERCA2a and PLN, and recombinant muscle acylphosphatase were incubated with antibodies against each of these proteins and the composition of the obtained immunoprecipitates was determined by Western blot analysis.

Materials and methods

Materials. All reagents were of analytical grade and were obtained from Sigma. Monoclonal (mouse) anti-SERCA2a ATPase antibody (clone 2A7-A1) was obtained from Affinity Bioreagents (Golden, CO), monoclonal (mouse) anti-phospholamban antibody (clone A1) was from Upstate biotechnology (Lake Placid, NY), and acylphosphatase recombinant form was obtained according to Modesti et al. [12]. Anti-acylphosphatase antibodies were raised in rabbit using the recombinant protein and purified by affinity chromatography [13]. Peroxidase coniugated anti-mouse or anti-rabbit secondary antibody was from Amersham. Super Signal enhanced chemiluminescent kit was from Pierce.

Preparation of SR vesicles (SRVs). Cardiac SRVs were isolated from rabbit heart according to Nediani et al. [14] and measured for protein [15].

Co-immunoprecipitation. SRVs were solubilized with 1% SDS in the immunoprecipitation buffer (10 mM Tris–HCl, pH 7, 150 mM NaCl, 1 mM EGTA, and 1 mM EDTA) at room temperature for 1 h and centrifuged for 15′ at 16,000*g*. The supernatants were rotated with 1% bovine serum albumin-treated G-Sepharose for 30′ and centrifuged to remove proteins bound non-specifically to G-Sepharose. The supernatants, at a final concentration of 0.5 mg/ml protein, were then incubated with monoclonal anti-SERCA2a or polyclonal anti-acylphosphatase or monoclonal anti-PLN antibody in the absence and in the presence of 5 or 10 μg of acylphosphatase recombinant form on ice for 1 h. The samples were rotated for 1 h with G-Sepharose and centrifuged and the pellets were washed three times in the immunoprecipitation buffer and solubilized for 10′ at 90 °C in Laemmli's sample buffer [16].

Western blot analysis of SERCA2a, phospholamban, and acylphosphatase. Samples were resolved by 10% and 15% SDS–PAGE and transferred to PVDF membranes. After blocking, the membrane were incubated with monoclonal anti-SERCA2a, or polyclonal anti-human muscle acylphosphatase or monoclonal anti-PLB antibody, washed three times in T-TBS solution (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20), and treated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody. The signals were detected with a chemiluminescence kit and the signal densities corresponding to SERCA2a or PLN immunoprecipitated were quantified by laser densitometry using Quantity One software (Bio-Rad laboratories).

Results and discussion

Taken together, the results of this study agree with the possibility that acylphosphatase, owing to its conformational properties, displaces PLN from SERCA2a. In this connection, we have previously demonstrated a physical interaction between SERCA2a and acylphosphatase since in controlled immunoprecipitation experiments the two proteins were co-precipitated by a monoclonal anti-SERCA2a antibody. In the same study, however, we found that thermally denaturated acylphosphatase was not more precipitated, suggesting that the maintenance of the native conformation of this enzyme was required for its binding to SERCA2a [8]. Now, in addition to confirming a physical association between SERCA2a and acylphosphatase, the present results also indicate that this binding could interfere with SERCA2a–PLN interaction. This, at least, emerges from the experiments that we performed by incubating SERCA2a and PLN (in the form of solubilized cardiac SR vesicles) with monoclonal anti-SERCA2a antibody in the absence and in the presence of increasing amounts of purified recombinant acylphosphatase. As shown in the Fig. 1A, when acylphosphatase was lacking Western blot analysis of the immunoprecipitate revealed two clear bands at 110 kDa and 25–27 kDa, corresponding, respectively, to SERCA2a and to the PLN pentameric form: a result which was expected since the well-known interaction between these proteins. On the other hand, in the presence of acylphosphatase, the analysis of the immunoprecipitate showed another band at 11 kDa, suggesting the co-immunoprecipitation of this enzyme together with the other two proteins. Moreover, as

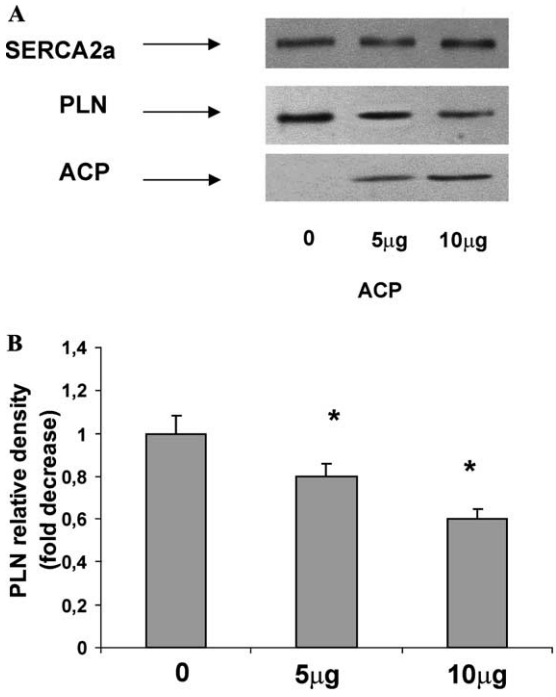

Fig. 1. Effect of acylphosphatase (ACP) on co-immunoprecipitation of SERCA2a with PLN using monoclonal anti-SERCA2a antibody. Solubilized SRVs were incubated with monoclonal anti-SERCA2a antibody as described under "Materials and methods" in the absence (0) and in presence of 5 or 10 μg of ACP. Immunoprecipitates were analyzed by Western blotting (A) and PLN signals were quantified by densitometric analysis (B). Each bar represents the mean value ± SD of four different blots. $*p < 0.05$ vs 0 by Student's t test.

indicated by densitometric analysis (Fig. 1B), the amount of PLN found in the immunoprecipitated significantly decreased with increasing acylphosphatase concentrations, with a diminution that, compared to the control values, was 17% for the lower (5 μg) and 37% for the higher acylphosphatase levels (10 μg). Besides these findings that clearly strengthen the supposed displacement of phospholamban by acylphosphatase, also other results of the present study support such an interpretation. In fact, when the above described mixtures of solubilized heart SR vesicles plus acylphosphatase were added with anti-acylphosphatase antibody (Fig. 2A) only this enzyme and SERCA2a were co-immunoprecipitated and the amount of SERCA2a detected in the precipitate increased significantly with the increase in acylphosphatase concentration. While, on one hand, these data corroborate the view of a selective interaction between acylphosphatase and SERCA2a, on the other hand the constant absence of PLN in the immunoprecipitate obtained with anti-acylphosphatase antibody strongly suggests that acylphosphatase, through a sort of competitive binding with SERCA2, can effectively displace PLN from its association with heart SR Ca^{2+} pump. Also the results obtained with monoclonal anti-phospholamban antibody agree with this conclusion. In fact, as shown in the Fig. 3, the amount of SERCA2a, which was co-immunoprecipitated with PLN by this antibody, was markedly reduced when 10 μg of acylphosphatase was added to the reaction mixture. The presence of acylphosphatase also resulted in an increased amount of PLN in the precipitate; a finding that may be explained admitting, as suggested by other authors [17], that the interaction with SERCA2a prevents, to a certain extent, PLN from reaching with its own antibody: this hindrance could be removed in consequence of the displacement produced by acylphosphatase. Much attention has been directed to SERCA2a–PLN interactions which, by affecting intracellular Ca^{2+} homeostasis, play a key role in the regulation of cardiac contractility and relaxation [2,3,17]. In this regard, recent studies reported that overexpression of a superinhibitory PLN mutant induces cardiac hypertrophy in mouse, impairing contractility and relaxation of heart muscle [18,19]; on the contrary, PLN ablation or overexpression of SERCA2a mutant lacking the functional association with PLN resulted in both

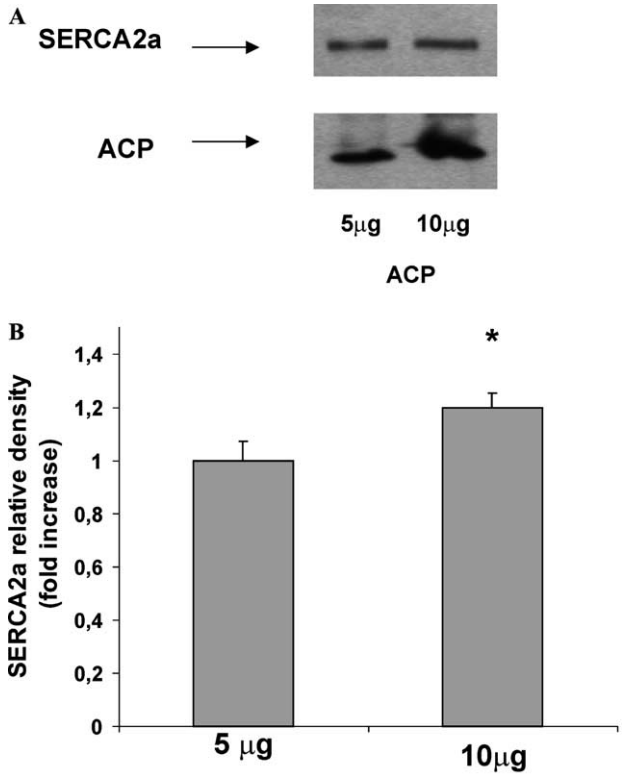

Fig. 2. Co-immunoprecipitation of SERCA2a and acylphosphatase (ACP) with polyclonal anti-ACP antibody. Solubilized SRVs were incubated with polyclonal anti-ACP antibody as described under "Materials and methods" in the presence of 5 and 10 μg of ACP. Immunoprecipitates were analyzed by Western blotting (A) and SERCA2a signals were quantified by densitometric analysis (B). Each bar represents the mean value ± SD of four different blots. $*p < 0.05$ vs 5 μg by Student's t test.

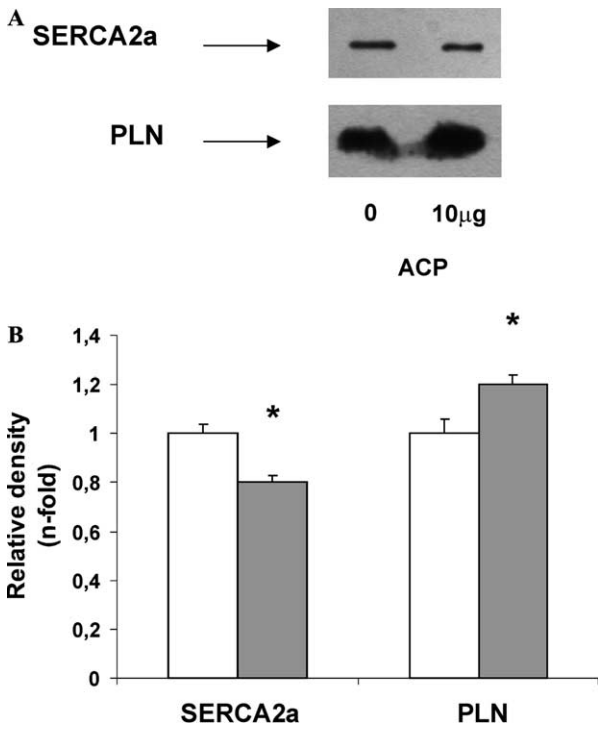

Fig. 3. Effect of acylphosphatase (ACP) on co-immunoprecipitation of SERCA2a with PLN using monoclonal anti-PLN antibody. Solubilized SRVs were incubated with monoclonal anti-SERCA2a antibody as described under "Materials and methods" in the absence (0) and in the presence of 10 μg of ACP. Immunoprecipitates were analyzed by Western blotting (A) and SERCA2a and PLN signals were quantified by densitometric analysis (B). Each bar represents the mean value ± SD of four different blots. □, (0); ■, 10 μg of ACP. $*p < 0.05$ vs 0 by Student's t test.

attenuation of cardiac hypertrophy and enhancement of cardiac contractility in a mouse dilated cardiomyopathy model [20] and in a mice pressure-overload model [21], suggesting that the removal of PLN inhibition on SERCA2a may be a new strategy to treat and to prevent heart failure. In this context it is our opinion that, although preliminary, the present data may be of special interest because they suggest a novel physiological mechanism which could contribute to modulate SERCA–PLN interactions, all the more so since in a recent study we found that, in a model of volume overload, increased acylphosphatase expression was associated to a recovery of SR function and an improved heart contractility [22].

On the basis of these considerations we think that it would be of interest to probe more deeply the molecular basis of the interaction between SERCA2a and acylphosphatase. More in particular, further studies will be aimed, in our intention, at identifying the residues that are involved in this interaction and to determine, with site-specific mutagenesis, the mutations that could cause a loss of the native conformation of acylphosphatase and a reduction of its hypothesized displacement effect.

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