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*Original Citation:*

A novel interaction mechanism accounting for different acylphosphatase effects on cardiac and fast twitch skeletal muscle sarcoplasmic reticulum calcium pump / C. Nediani; C. Fiorillo; S. Rigacci; F. Magherini; M. Francalanci; G. Liguri; A. Pacini; P. Nassi. - In: FEBS LETTERS. - ISSN 0014-5793. - STAMPA. - 443:(1999), pp. 308-312. [10.1016/S0014-5793(98)01717-7]

*Availability:*

The webpage <https://hdl.handle.net/2158/308784> of the repository was last updated on 2016-11-18T10:18:19Z

*Published version:*

DOI: 10.1016/S0014-5793(98)01717-7

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# A novel interaction mechanism accounting for different acylphosphatase effects on cardiac and fast twitch skeletal muscle sarcoplasmic reticulum calcium pumps

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Received 2 November 1998; received in revised form 17 December 1998

**Abstract** In cardiac and skeletal muscle  $\text{Ca}^{2+}$  translocation from cytoplasm into sarcoplasmic reticulum (SR) is accomplished by different  $\text{Ca}^{2+}$ -ATPases whose functioning involves the formation and decomposition of an acylphosphorylated phosphoenzyme intermediate (EP). In this study we found that acylphosphatase, an enzyme well represented in muscular tissues and which actively hydrolyzes EP, had different effects on heart (SERCA2a) and fast twitch skeletal muscle SR  $\text{Ca}^{2+}$ -ATPase (SERCA1). With physiological acylphosphatase concentrations SERCA2a exhibited a parallel increase in the rates of both ATP hydrolysis and  $\text{Ca}^{2+}$  transport; in contrast, SERCA1 appeared to be uncoupled since the stimulation of ATP hydrolysis matched an inhibition of  $\text{Ca}^{2+}$  pump. These different effects probably depend on phospholamban, which is associated with SERCA2a but not SERCA1. Consistent with this view, the present study suggests that acylphosphatase-induced stimulation of SERCA2a, in addition to an enhanced EP hydrolysis, may be due to a displacement of phospholamban, thus to a removal of its inhibitory effect.

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**Key words:** Heart sarcoplasmic reticulum  $\text{Ca}^{2+}$  pump; Phospholamban; Acylphosphatase

## 1. Introduction

In both cardiac and skeletal muscle active calcium transport by sarcoplasmic reticulum (SR) is mediated by an ATPase enzyme of about 100 kDa which serves as an energy transducer and a translocator of calcium ions.  $\text{Ca}^{2+}$  transport and ATP hydrolysis are coupled through a complex series of elementary steps involving the formation and the decomposition of a phosphoenzyme intermediate (EP) [1], which has been identified as an acylphosphate, since phosphorylation takes place at a carboxyl group of an aspartic acid residue [2,3]. Cardiac SR  $\text{Ca}^{2+}$ -ATPase (SERCA2a), unlike the analogous skeletal enzyme (SERCA1), is regulated by a 22 kDa integral

membrane protein named phospholamban (PLN), whose phosphorylation by cAMP-dependent protein kinase (PKA) leads to an increase in the rate of active  $\text{Ca}^{2+}$  transport. Thus, unphosphorylated PLN is believed to function as a calcium pump inhibitor that becomes inactive upon phosphorylation [4–6]. Many laboratories that have studied the effect of PKA-catalyzed phosphorylation of PLN on the kinetics of calcium uptake or  $\text{Ca}^{2+}$ -ATPase activity have reported a decrease in the  $K_{\text{mCa}^{2+}}$  or an increase in  $V_{\text{maxCa}^{2+}}$  or both these effects [7–13].

Acylphosphatase (EC 3.6.1.7) is a widespread cytosolic enzyme, well represented in skeletal and heart muscle, that catalyzes the hydrolysis of the carboxylphosphate bond of acylphosphates such as 3-phosphoglyceroyl phosphate [14], carbamoyl phosphate [15] and succinoyl phosphate [16]. In addition to the above soluble, low molecular weight substrates we found that this enzyme hydrolyzed the phosphorylated intermediates of various transport ATPases, notably those of erythrocyte membrane [17] and heart sarcolemma  $\text{Ca}^{2+}$ -ATPase [18]. More recently we reported that acylphosphatase actively hydrolyzed the phosphoenzyme of the cardiac SR  $\text{Ca}^{2+}$  pump, with an apparent  $K_{\text{m}}$  in the order of  $10^{-7}$  M, suggesting a high affinity of our enzyme for this special substrate [19]. This caused us to study the effect of acylphosphatase on the functional properties of the heart SR  $\text{Ca}^{2+}$  pump. The results obtained indicated that in unphosphorylated cardiac SR vesicles acylphosphatase enhanced the rate of ATP hydrolysis and  $\text{Ca}^{2+}$  uptake. On the other hand, in vesicles whose phospholamban was PKA-phosphorylated, acylphosphatase still stimulated the rate of  $\text{Ca}^{2+}$  uptake and ATP hydrolysis but to a lesser extent. As an interpretation of these findings we proposed that acylphosphatase, in addition to the stimulatory effect due to its catalytic activity, could also act through the removal of phospholamban inhibition. In fact acylphosphatase, a small protein of about 11 kDa, contains a structural motif where 12 residues (from position 55 to 66) form an amphipathic  $\alpha$ -helix with a prevalence of basic groups [20]. This structure resembles that of the phospholamban cytoplasmic 1A domain [21] which is essential for the association of phospholamban with SERCA2a, so we supposed that acylphosphatase could interact with this transport system taking the place of unphosphorylated phospholamban whose inhibitory effect would therefore be removed.

The present paper reports the studies that we performed in order to verify the above hypothesis and to probe more deeply the interactions of acylphosphatase with the heart SR  $\text{Ca}^{2+}$  pump.

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**Abbreviations:** SR, sarcoplasmic reticulum; SRV, SR vesicle; SERCA, sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase; EP, phosphoenzyme; PKA, cAMP-dependent protein kinase; PLN, phospholamban

## 2. Materials and methods

PKA from rabbit muscle, Tris-ATP, protein G-Sepharose 4B Fast flow were from Sigma.

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and  $^{45}\text{CaCl}_2$  were obtained from DuPont NEN. Monoclonal (mouse) anti-SERCA2a ATPase antibody (IgG2a) (Clone 2A7-A1, ascites fluid) was from Affinity Bioreagents, Inc. Anti-mouse IgG (donkey) peroxidase conjugate was from Amersham, while anti-rabbit IgG (goat) peroxidase conjugate was from Calbiochem. Nitrocellulose filters and membranes were obtained from Sartorius. Enhanced chemoluminescence assay (ECL) was from Amersham.

### 2.1. Acylphosphatase: purification and some properties

Acylphosphatase was purified from bovine heart according to Ramponi et al. [22] for the extraction, and according to Stefani et al. for the other steps [23]. The acylphosphatase Asn-41 mutant to Ser, Asn41Ser, was obtained by site-directed mutagenesis and purified by Modesti et al. [24]. The properties of this mutant are reported in Section 3. Anti-acylphosphatase antibodies were raised in rabbits using the recombinant protein [24] and purified by affinity chromatography [25].

### 2.2. Preparation of SR vesicles (SRVs)

Cardiac and skeletal SRVs were isolated from heart and fast twitch skeletal muscle (adductor magnus) of rabbit according to Jones et al. [26] and measured for protein by the method of Bradford [27]. To induce phospholamban phosphorylation cardiac SRVs (0.5 mg/ml) were incubated in 40 mM Tris-HCl, pH 7.4, 120 mM KCl, 5 mM  $\text{MgCl}_2$ , 5 mM Tris-ATP with 10  $\mu\text{M}$  cAMP and 1 mg/ml PKA at 25°C for 10 min. The reaction was terminated in ice. PLN phosphorylation was verified as previously described [19].

### 2.3. Preparation of phosphorylated intermediate of cardiac SR

#### $\text{Ca}^{2+}$ -ATPase and its incubation with acylphosphatase

Cardiac SRVs were phosphorylated by  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  according to Nediani et al. [19]. 0.15 mg/ml of phosphorylated SRVs was incubated in 30 mM Tris-HCl, pH 7.4 at 37°C for 30 s without and with differing amounts of acylphosphatase. The reaction was stopped with 10% ice-cold trichloroacetic acid and the suspension was centrifuged at 13000 $\times g$  for 5 min. Aliquots of the supernatants were taken to measure free  $^{32}\text{P}$  radioactivity.

### 2.4. ATPase activity measurements

$\text{Ca}^{2+}$ -ATPase activity was assayed as previously described [19]. In our experimental conditions a free  $\text{Ca}^{2+}$  concentration of approximately 10  $\mu\text{M}$  was calculated using the equations of Katz et al. [28]. The reaction was started by the addition of ATP or of an aliquot of the vesicle suspension and stopped after 10 min with one volume of ice-cold 20% trichloroacetic acid. Phosphate release from ATP was measured by the malachite green procedure [29] in aliquots of the supernatant.

### 2.5. $\text{Ca}^{2+}$ influx measurements into SRVs

For these measurements the reaction mixture was the same as for ATPase assays except that it included  $^{45}\text{CaCl}_2$  and 5 mM oxalate.

After 30 s of incubation at 37°C, the vesicles were separated from the medium by filtration through a Millipore filter (0.45  $\mu\text{m}$  pore size), and then the filter was immediately washed two times with 4 ml of ice-cold 20 mM Tris-HCl, pH 7.4, 1 mM EGTA, and 100 mM KCl. Oxalate-facilitated  $^{45}\text{Ca}$  uptake was measured as the difference in  $^{45}\text{Ca}$  influx into vesicle at time zero and at the end of incubation. Radioactivity trapped on the filter was determined by liquid scintillation spectroscopy.

### 2.6. Coimmunoprecipitation and Western blot analysis of SERCA2a and acylphosphatase

Cardiac specimens were homogenized in three volumes of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1% Triton, 1 mM EGTA, 1 mM EDTA) with an Ultraturrax apparatus for 90 s and then centrifuged at 36000 $\times g$  for 30 min to remove insoluble material. Solubilized protein fraction was incubated for 1 h in ice with 5  $\mu\text{l}$  of monoclonal anti-SERCA2a antibody in the presence of the Asn41Ser mutant of acylphosphatase or of its denatured form. Immune complexes were collected on protein G-Sepharose and washed four times in the immunoprecipitation buffer. Samples were resolved by 8% and 15% SDS-PAGE and transferred to nitrocellulose membranes [30]. Immunorecognition was performed with monoclonal (mouse) anti-SERCA2a antibody (1:1500), polyclonal (rabbit) anti-human muscle acylphosphatase antibody (1:4000) and secondary peroxidase-conjugated specific antibodies. Immunoreactive bands were visualized by ECL detection reagent on a Biomax Light-1 film (Kodak).

## 3. Results

Acylphosphatase used in the present study was a pure product which had a specific activity of 3650 U/mg protein using benzoyl phosphate as substrate [31]. The Asn41Ser acylphosphatase mutant was almost completely devoid of catalytic activity (specific activity 10 U/mg protein using the above substrate) but retained substrate binding ability and unchanged three-dimensional structure with respect to the active enzyme [32].

### 3.1. Effect of acylphosphatase on the phosphorylated intermediate of cardiac SR $\text{Ca}^{2+}$ -ATPase

Fig. 1 shows the phosphate release from cardiac SR  $\text{Ca}^{2+}$ -ATPase phosphoenzyme in the absence and in the presence of differing concentrations of acylphosphatase. These acylphosphatase/SRV protein ratios were chosen because they are within the physiological range which, in the heart and in the skeletal muscle, was estimated to be 80–130 U/mg of SRV protein [33]. When acylphosphatase was present the release of phosphate was always higher than the spontaneous hydroly-

Table 1  
Effect of acylphosphatase on cardiac and skeletal SR  $\text{Ca}^{2+}$ -ATPase activity

Acylphosphatase (U/mg SRC protein)	Cardiac SR $\text{Ca}^{2+}$ -ATPase activity (nmol/min/mg SRV protein)	Increase (n-fold)	Skeletal SR $\text{Ca}^{2+}$ -ATPase activity ( $\mu\text{mol}/\text{min}/\text{mg}$ SRV protein)	Increase (n-fold)
0	66.67 $\pm$ 6.21		1.81 $\pm$ 0.09	
10	76.58 $\pm$ 8.46	1.14	2.03 $\pm$ 0.22	1.12
20	85.92 $\pm$ 8.33	1.28	2.25 $\pm$ 0.15	1.24
30	95.39 $\pm$ 7.47	1.43	2.57 $\pm$ 0.24	1.42
40	107.67 $\pm$ 8.79	1.61	2.80 $\pm$ 0.13	1.54
50	116.09 $\pm$ 9.90	1.74	2.98 $\pm$ 0.07	1.64
75	129.04 $\pm$ 14.12	1.93	3.16 $\pm$ 0.18	1.74
100	147.11 $\pm$ 14.68	2.20	3.39 $\pm$ 0.27	1.87
Asn41Ser	94.67 $\pm$ 13.35	1.41	1.77 $\pm$ 0.07	–
hd-Asn41Ser	68.53 $\pm$ 5.81	1.02	1.73 $\pm$ 0.09	–

Values are means  $\pm$  S.E.M. of six replicate determinations. Changes observed with differing amounts of acylphosphatase were statistically significant for both cardiac and skeletal  $\text{Ca}^{2+}$ -ATPase activity ( $P < 0.01$  by one-way analysis of variance). hd: heat-denatured.

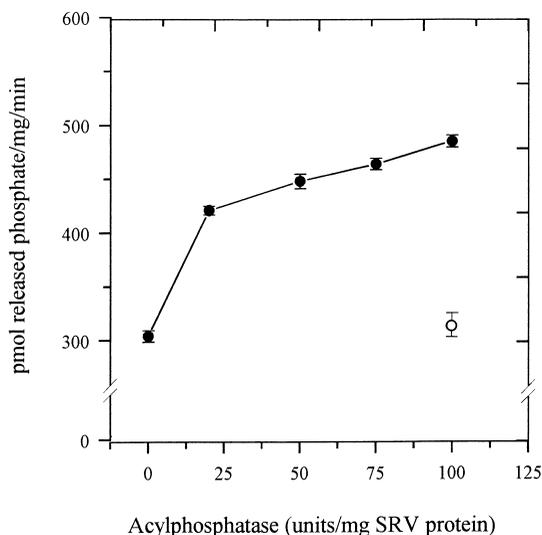


Fig. 1. Effect of different acylphosphatase concentrations on cardiac SR  $\text{Ca}^{2+}$ -ATPase phosphoenzyme. The acylphosphatase mutant Asn41Ser ( $\circ$ ) was added at a concentration corresponding to 100 U active enzyme/mg of SRV protein. Each point is the mean  $\pm$  S.E.M. of five experiments performed on different vesicle preparations. All the changes in phosphate release induced by acylphosphatase were statistically significant ( $P < 0.01$  by one-way analysis of variance).

ysis, even at the lowest enzyme concentration, and rose significantly with increasing acylphosphatase/SRV protein ratio. In contrast, no significant enhancement of phosphate release was observed using the Asn41Ser mutant of acylphosphatase at a concentration corresponding to 100 U of active enzyme per mg of SRV.

### 3.2. Coimmunoprecipitation between cardiac $\text{Ca}^{2+}$ -ATPase and the Asn41Ser mutant of acylphosphatase

When the cardiac solubilized protein fraction, used as a source of  $\text{Ca}^{2+}$ -ATPase, and the Asn41Ser mutant of acylphosphatase were incubated together, in the presence of monoclonal anti-SERCA2a antibody, Western blot analysis of the immunoprecipitate (Fig. 2) revealed two clear bands at 110 kDa and 11 kDa, corresponding to SERCA2a and to the acylphosphatase mutant, which indicates coimmunoprecipitation of these proteins. Only one band at 110 kDa was observed when the above incubation was performed using the heat-denatured form of the mutant. No positive band was evident when anti-SERCA2a antibody was incubated only with Asn41Ser, so immunoreactivity between these two proteins could be excluded.

Table 2  
Effect of acylphosphatase on cardiac and skeletal SR  $\text{Ca}^{2+}$  uptake

Acylphosphatase (U/mg SRV protein)	Cardiac SR $\text{Ca}^{2+}$ uptake (nmol/min/mg SRV protein)	Skeletal SR $\text{Ca}^{2+}$ uptake ( $\mu\text{mol}/\text{min}/\text{mg}$ SRV protein)
0	80.82 $\pm$ 4.40	2.10 $\pm$ 0.05
20	103.85 $\pm$ 8.25	1.51 $\pm$ 0.02
50	131.54 $\pm$ 6.75	0.96 $\pm$ 0.01
75	161.87 $\pm$ 7.42	0.73 $\pm$ 0.04
100	172.31 $\pm$ 8.83	0.58 $\pm$ 0.03
Asn41Ser	129.72 $\pm$ 5.68	1.97 $\pm$ 0.07
hd-Asn41Ser	83.42 $\pm$ 3.76	2.03 $\pm$ 0.04

Values are means  $\pm$  S.E.M. of six replicate determinations. Changes observed with different amounts of acylphosphatase were statistically significant for both cardiac and skeletal  $\text{Ca}^{2+}$  uptake ( $P < 0.01$  by one-way analysis of variance). hd: heat-denatured.

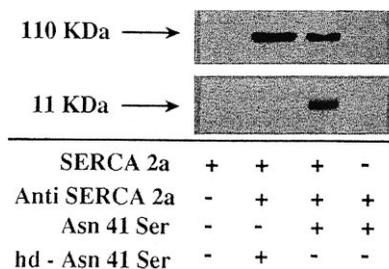


Fig. 2. Coimmunoprecipitation of SERCA2a (110 kDa) and the acylphosphatase mutant Asn41Ser (11 kDa) by monoclonal anti-SERCA2a antibody. Immunoprecipitates were subjected to SDS polyacrylamide gel electrophoresis and Western blot analysis using either an anti-SERCA2a antibody or an anti-acylphosphatase antibody.

### 3.3. Effect of acylphosphatase on cardiac and skeletal SR $\text{Ca}^{2+}$ -ATPase activity and $\text{Ca}^{2+}$ transport

Table 1 shows that, as expected from our previous studies [17,18], acylphosphatase significantly stimulated the rates of  $\text{Ca}^{2+}$ -dependent ATP hydrolysis of both cardiac and skeletal SR vesicles. With 100 U acylphosphatase per mg SRV protein, the concentration that gave maximal stimulation, the increase over the control value was 120% for cardiac and 87% for skeletal muscle  $\text{Ca}^{2+}$ -ATPase activity. In the presence of the Asn41Ser mutant of acylphosphatase, at the same protein concentration, cardiac  $\text{Ca}^{2+}$ -ATPase activity was still significantly increased (41% over the basal value), while skeletal  $\text{Ca}^{2+}$ -ATPase activity was unaffected. Using the heat-denatured Asn41Ser mutant the stimulatory effect on cardiac SRVs disappeared.

As regards  $\text{Ca}^{2+}$  uptake, acylphosphatase effects were markedly different depending on the use of cardiac or skeletal muscle SRVs. Acylphosphatase addition to cardiac SRVs resulted in a concentration-dependent enhancement of  $\text{Ca}^{2+}$  transport which paralleled the stimulation of  $\text{Ca}^{2+}$ -ATPase activity (Table 2). Also the Asn41Ser mutant (but not its heat-denatured form) stimulated the  $\text{Ca}^{2+}$  uptake by cardiac SRVs, although to a lesser extent than the native enzyme (60% over the control value against 100% obtained with the active form). In contrast, in skeletal SR vesicles  $\text{Ca}^{2+}$  uptake was gradually and significantly inhibited at increasing amounts of acylphosphatase while the Asn41Ser mutant did not exert any effect.

### 3.4. Effect of acylphosphatase on cardiac PKA-phosphorylated SR vesicles

Also in this case acylphosphatase induced a concentration-dependent increase in the rate of ATP hydrolysis and  $\text{Ca}^{2+}$

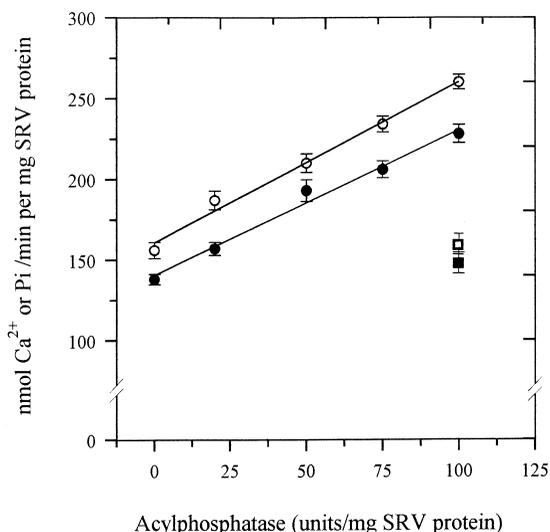


Fig. 3.  $\text{Ca}^{2+}$ -ATPase activity (●) and  $\text{Ca}^{2+}$  uptake (○) of cardiac PKA-phosphorylated SRVs as a function of acylphosphatase concentrations.  $\text{Ca}^{2+}$ -ATPase activity was expressed as nmol of ATP split/min per mg of SRV protein and  $\text{Ca}^{2+}$  uptake as nmol  $\text{Ca}^{2+}$  transported into vesicles/min per mg of SRV protein. Each point represents the mean value  $\pm$  S.E.M. of five determinations. Changes in  $\text{Ca}^{2+}$ -ATPase activity and  $\text{Ca}^{2+}$  transport, with increasing amounts of acylphosphatase, were statistically significant ( $P < 0.01$  by one-way analysis of variance). ■ and □, respectively, indicate the values obtained for  $\text{Ca}^{2+}$ -ATPase activity and  $\text{Ca}^{2+}$  uptake with the acylphosphatase mutant Asn41Ser at a concentration corresponding to 100 U of active enzyme/mg of SRV protein.

transport (Fig. 3), but these stimulatory effects were less marked (the maximal stimulation was about 60% over the control values) than those observed using unphosphorylated vesicles. No significant enhancement of the rate of  $\text{Ca}^{2+}$ -ATPase activity and  $\text{Ca}^{2+}$  transport was shown using the Asn41Ser mutant of acylphosphatase.

#### 4. Discussion

The main goal of the present study was to establish whether acylphosphatase, in addition to its hydrolytic activity on EP, could stimulate the cardiac SR  $\text{Ca}^{2+}$  pump through another mechanism, owing to its conformational properties. To verify this hypothesis we used a particular acylphosphatase mutant, Asn41Ser, which for the above reasons appeared an excellent tool for our purpose, all the more so since we observed that it was ineffective even on the phosphorylated intermediate of the cardiac SR  $\text{Ca}^{2+}$  pump.

That being established, our first attempt in the present study was to verify a physical interaction between acylphosphatase and heart SR  $\text{Ca}^{2+}$ -ATPase. In this connection, the results that we obtained in controlled immunoprecipitation experiments strongly suggest the possibility of a specific binding between these two proteins, which were selectively co-precipitated by a monoclonal anti-SERCA2a antibody. In the same conditions, the heat-denatured form of our mutant was not precipitated, thus its native conformation appeared to be required for the interaction with SR  $\text{Ca}^{2+}$ -ATPase.

Other experiments were designed to examine the effect of the Asn41Ser acylphosphatase mutant on the functional properties of SERCA2a, notably the rate of ATP hydrolysis and

$\text{Ca}^{2+}$  transport, and to compare these effects with those produced by the active enzyme. To explore the relationship between our enzyme and phospholamban in affecting the activity of  $\text{Ca}^{2+}$  pump, the above experiments were performed using unphosphorylated heart SRVs, the same vesicles whose phospholamban was PKA-phosphorylated, and fast twitch skeletal muscle SRVs where phospholamban was absent. The Asn41Ser acylphosphatase mutant significantly stimulated (although to a lesser extent than the native enzyme)  $\text{Ca}^{2+}$ -dependent ATP hydrolysis and  $\text{Ca}^{2+}$  transport in unphosphorylated cardiac SRVs, but it failed to exert this stimulatory effect when its three-dimensional structure was altered by thermal denaturation or when SRV phospholamban underwent PKA phosphorylation, an event which per se can remove phospholamban inhibition on the  $\text{Ca}^{2+}$  pump. Furthermore, the acylphosphatase mutant did not exhibit any effect on the activity of  $\text{Ca}^{2+}$ -ATPase in skeletal muscle SRVs lacking phospholamban. In our opinion, all these findings, together with the above-mentioned specific binding to SERCA2a, are consistent with the possibility that part of the stimulatory effect of acylphosphatase on the heart SR  $\text{Ca}^{2+}$  pump may be ascribed to a conformational interaction leading to a removal of phospholamban inhibition. Besides, the more marked enhancement of  $\text{Ca}^{2+}$ -ATPase activity induced by acylphosphatase in heart than in skeletal muscle SRVs suggests a stimulatory mechanism additional to the common effect of accelerated EP hydrolysis.

Other interesting results of the present study are the different effects of acylphosphatase on the functional properties of the SR  $\text{Ca}^{2+}$ -ATPases from heart and from fast twitch skeletal muscle. As a tentative hypothesis we propose that acylphosphatase can act at various steps of the reaction sequence involved in  $\text{Ca}^{2+}$  transport across SR, owing to the presence or the absence of phospholamban. In cardiac SRVs acylphosphatase-induced hydrolysis of EP would take place in the so-called  $\text{E}_2\text{P}$  form, that is when  $\text{Ca}^{2+}$  ions are already translocated, hence a parallel increase in the rate of ATP hydrolysis and of calcium transport. In contrast, in skeletal muscle SRVs, where phospholamban is absent, acylphosphatase would act on the so-called  $\text{E}_1\text{P}$  form, in other words before the  $\text{Ca}^{2+}$  ions are translocated: this would result in the observed 'uncoupling' effect, namely a stimulation of ATP hydrolysis, with a concomitant inhibition of  $\text{Ca}^{2+}$  transport. The results of our previous studies [17,18], indicating that acylphosphatase had a similar uncoupling effect on other  $\text{Ca}^{2+}$  pumps lacking phospholamban (red cell membrane and heart sarcolemma  $\text{Ca}^{2+}$ -ATPases), might support this view.

*Acknowledgements:* This work was supported by grants from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (Progetto Nazionale Insufficienza Cardiaca) and from the University of Florence (Fondi di Ateneo, ex quota 60%). We are grateful to Dr. Vanessa Ponziani for her technical assistance and to Dr. Niccolò Taddei for the gift of the purified acylphosphatase mutant, Asn41Ser.

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