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ACYLPHOSPHATASE STIMULATES Ca^{2+} TRANSPORT AND Ca^{2+}-DEPENDENT ATPase ACTIVITY IN CARDIAC SARCOPLASMIC RETICULUM

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SUMMARY. Acylphosphatase purified from heart muscle actively hydrolyzes the phosphoenzyme intermediate of cardiac sarcoplasmic reticulum Ca^{2+}-ATPase. This effect was evident with acylphosphatase concentrations (up to 100 units/mg sarcoplasmic reticulum protein) that fall within the physiological range, and the low value of the apparent Km, on the order of 10^{-7}M, suggests a high affinity towards this special substrate. Moreover, acylphosphatase addition to sarcoplasmic reticulum vesicles significantly enhanced the rate of Ca^{2+}-dependent ATP hydrolysis. Maximal stimulation, observed with 100 units/mg vesicular protein, resulted in an ATPase activity which was about two folds over basal value. The same acylphosphatase concentration increased at a similar extent the rate of ATP driven Ca^{2+} influx into sarcoplasmic reticulum vesicles. Taken together these findings lead to suppose that acylphosphatase, owing to its hydrolytic activity, induces an accelerated turnover of the phosphoenzyme intermediate, whence an overall stimulation of heart sarcoplasmic reticulum Ca^{2+} pump, affecting both ATP hydrolysis and Ca^{2+} influx.

Key words: Acylphosphatase and heart sarcoplasmic reticulum Ca^{2+} pump

INTRODUCTION

Together with the activity of two sarcolemmal systems, namely the Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger [1], active Ca^{2+} transport into sarcoplasmic reticulum (SR) plays a central role in the excitation-contraction coupling of heart muscle. In fact, cardiac SR contains a Ca^{2+}-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3.) which acts as a Ca^{2+} pump, transducing chemical energy of ATP into a gradient of calcium ions across SR membrane [2]. Ca^{2+} translocation is tightly coupled with ATP hydrolysis that proceeds through a series of reactions involving the formation and the decomposition of a phosphoenzyme (EP) intermediate [3]. As for other transport ATPases, the EP intermediate of SR Ca^{2+}-ATPase was recognized to be an acylphosphate since phosphorylation was found to occur at a carboxyl group of an aspartic acid residue [4]. Changes in the rates of the elementary reactions at which EP is formed and decomposed appear to represent the action mechanism for the effect of phospholamban that is assumed to serve as the main physiological regulator of heart SR Ca^{2+}-ATPase [5],.

Abbreviations: SR, sarcoplasmic reticulum; EP, the phosphorylated form of Ca^{2+}-ATPase, Tris, tris (hydroxymethyl)-aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; EGTA, [ethylenbis(oxyethylenenitrilo)]tetracetic acid.

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Acylphosphatase (EC 3.6.1.7.), a widespread enzyme well represented in heart muscle [6], catalyzes the hydrolysis of the carboxylphosphate bond of acylphosphates. For several years we have been investigating structural and functional properties of acylphosphatase purified from various animal tissues. More recently we have found that this enzyme, in addition to soluble substrates such as 3-phosphoglyceroylphosphate or carbamoylphosphate, can act on the acylphosphorylated intermediates of some transport ATPases, notably on those of erythrocyte membrane [7], and of heart sarcolemma Ca^{2+}-ATPase [8].

Aim of the present study was to evaluate whether a similar effect of acylphosphatase on the EP intermediate of heart SR Ca^{2+}-ATPase resulted in modified functional properties of this important calcium pump.

MATERIALS AND METHODS

Tris-ATP, potassium-oxalate, were from Sigma Chimica (Milano, Italy). [γ-^{32}P]ATP (3000Ci/mmol) and $^{45}CaCl_2$ (29.77 mCi/mg) were purchased from Du Pont NEN (Brussel, Belgium). Nitrocellulose filters (0.45 μm) were obtained from Sartorius (Firenze, Italy). All other chemicals were of analytical grade.
Acylphosphatase was purified to homogeneity from bovine heart according to Ramponi *et al.* [9] for the extraction and according to Stefani *et al.* [10] for the other steps. The enzyme, isolated as a pure product, had a specific activity of 3650 units/mg protein using benzoyl phosphate as substrate [11]. Benzoyl phosphate was synthesized as per Camici *et al.* [12]. Cardiac SRV protein content was assayed by the method of Beisenherz *et al.* [13].

Preparation of cardiac sarcoplasmic reticulum vesicles (SRVs).

Cardiac SRVs were isolated from trimmed calf ventricles by the method of Jones *et al.*[14] except that the Ca^{2+}-oxalate loading step was omitted. The final pellet was resuspended in 0.3M KCl, 0.3M sucrose, 20mM Hepes pH 7 and stored frozen at -80 °C. A yield of 55-65 mg of vesicle protein per 100 g of wet tissue was routinely obtained.
Na^+,K^+-ATPase activity (see below) was measured to determine the extent of contamination of SR fraction by sarcolemma.

ATPase assays

All ATPase activities were determined at 37 °C by measuring inorganic phosphate (Pi) liberation. Reactions were started by the addition of ATP or of an aliquot of the vesicle suspension, and stopped after 10 min with one volume of 20% ice-cold trichloroacetic acid. After centrifugation at 12500×g for 5 min aliquots were taken from the supernatant and Pi was measured using a malachite green procedure, according to Baykov *et al.* [15]. For Ca^{2+}-ATPase, total activity was determined in a medium containing 50mM Tris-HCl pH 7.4, 3mM $MgCl_2$, 100mM KCl, 5mM NaN_3 (which inhibits mitochondrial but not SR Ca^{2+}-ATPase), 50μM $CaCl_2$, 3mM ATP, and 50μg/ml vesicle protein. The basal ATPase activity, measured in presence of 1mM Tris-EGTA in place of $CaCl_2$, was subtracted from the total ATPase to give Ca^{2+}-dependent ATPase activity, which was expressed as nmol/min per mg SRV protein. In the Ca^{2+}-ATPase assays with added $CaCl_2$ and no EGTA, the free Ca^{2+} concentration, determined primarily by the calcium binding of ATP, was calculated as 10μM according to Katz *et al.* [16].
Na^+,K^+-ATPase was assayed in a medium containing 50mM Tris-HCl pH 7.4, 3mM $MgCl_2$, 100mM NaCl , 1mM Tris-EGTA, 100mM KCl, 3mM ATP and 50 μg/ml vesicle protein. Na^+,K^+-ATPase activity was calculated as the difference in the rate of ATP hydrolysis without and with 1mM ouabain.

Preparation of phosphorylated intermediate of SR Ca^{2+}-ATPase

SRVs were phosphorylated according to Beekman *et al.* [17] with slight modifications. The reaction mixture contained 30mM Tris-HCl pH 7, 1mM $MgCl_2$, 120mM KCl, [γ-^{32}P] ATP (0.2 mCi/μmol), 125μM $CaCl_2$ or 1 mM EGTA and 1mg of cardiac SR vesicles in a total volume of 1 ml. The reaction was started by the addition of non radioactive ATP to give a final concentration of 10 μM and terminated after 30 s

with 4 ml of ice-cold 6% trichloroacetic acid containing 1mM ATP and 5mM NaH_2P0_4. Following centrifugation at 30000xg for 10 min, the supernatant was decanted and the pellet was washed once with the above trichloroacetic acid mixture and two more times with 0.15M Tris-HCl pH 7.4. The final pellet was resuspended in 30mM Tris-HCl pH 7.4 and aliquots were assayed for radioactivity and protein content. The level of phosphoenzyme was taken as the difference between the amount of ^{32}P incorporated into vesicles in presence of $CaCl_2$ or EGTA. 1 mg/ml of phosphorylated vesicles were incubated in 30 mM Tris-HCl pH 7.4 at 37 °C for 30 sec with and without differing amounts of acylphosphatase. The reaction was stopped with one volume of 10% ice-cold trichloroacetic acid and the suspensions were centrifuged at 13000xg for 5 min. Aliquots of the supernatant were taken to measure free ^{32}P radioactivity. In another series of experiments differing amounts of phosphorylated membranes were incubated with a fixed amount of acylphosphatase (100 units). Controls for spontaneous hydrolysis were incubated under the same conditions for each concentration of phosphorylated vesicles and subtracted to give acylphosphatase-induced phosphate release.

Assay for Ca^{2+}influx into SRVs.

The incubation mixture was the same as for ATPase assays except that it included $^{45}CaCl_2$ (5μCi/μ mol) and 5mM potassium-oxalate. Active $^{45}Ca^{2+}$ transport was measured as the difference in $^{45}Ca^{2+}$ influx into vesicles in the presence and in the absence of 3mM Tris-ATP which was used to start the reaction. After 30 s the reaction mixture was filtered through a Millipore filter (0.45 μm pore size) which was immediately washed two times with 4 ml of ice-cold 20mM Tris-HCl pH 7.4, 1mM EGTA and 0.1M HCl. Radioactivity trapped on the filter was determined by liquid scintillation spectroscopy.

Expression of the results.

Data presented under Results are the mean of several determinations. For curve drawing and kinetics constant calculation the Fig.P computer program by Biosoft (Cambridge, U.K.) was used. Statistical analysis was performed by one-way analysis of variance.

RESULTS

Preparations used in these studies were highly enriched in sarcoplasmic reticulum vesicles, as judged from the efficiency of their typical activities such as Ca^{2+}uptake, Ca^{2+}-dependent ATPase and Ca^{2+}-dependent phosphoenzyme intermediate formation. Sarcolemma contamination of our SRVs was negligible, since Na^+,K^+-ATPase activity was absent. SRVs vesicles incubated in presence of [γ-^{32}P] ATP and Ca^{2+}, as described under Materials and Methods, formed a Ca^{2+}-dependent EP intermediate, whose level, after subtracting non specific ^{32}P bound in presence of EGTA instead of Ca^{2+}, was 145 pmol ^{32}P bound/mg vesicle protein, a value which agrees with that reported by other authors [17].

Table 1 shows the phosphate release from SR Ca^{2+}-ATPase phosphoenzyme observed in the absence and in the presence of differing concentrations of acylphosphatase. These acylphosphatase/SRV protein ratios were chosen because they are within the limits of the physiological range which, in heart muscle, was estimated to be from 80 to 130 units/mg of SRV protein [6]. In presence of acylphosphatase the phosphate release, net of spontaneous hydrolysis, was evident even at the lowest enzyme concentrations and rose significantly with the increase in acylphosphatase/SRV protein ratio; the maximal effect was observed with 100 units/mg SRV protein. No significant enhancement of phosphate release occurred using higher concentration of the enzyme.

In order to characterize kinetically the effect of acylphosphatase, variable amounts of phosphorylated vesicles were incubated with a fixed amount of acylphosphatase and acylphosphatase-induced hydrolysis was measured as a function of EP concentration. In these conditions the initial rate of enzymatic

Table 1
Effect of different acylphosphatase concentrations on the Ca^{2+}-ATPase phosphorylated intermediate from cardiac sarcoplasmic reticulum vesicles (SRVs).

Acylphosphatase (units/mg SRV protein)	Phosphate release (pmol/min)
none	149.72 ± 0.78
25	200.25 ± 3.43
50	249.29 ± 5.22
100	282.72 ± 3.94
200	261.05 ± 2.01

Labeled vesicles were incubated in 30mM Tris-HCl pH 7.4, at 37 °C for 30 sec with varying amounts of acylphosphatase. The phosphoenzyme level was calculated as 145 pmol phosphate bound/mg vesicle protein. Each point is the mean ± S.E.M. of five experiments. All the changes in phosphate release induced by acylphosphatase were statistically significant ($p<0.01$ by the one-way analysis of variance).

dephosphorylation rose with the increase of EP concentration along a hyperbolic curve, with an apparent Km of 157.08 ± 19.6 nM (Figure 1).

To see whether the above action of acylphosphatase on EP modified the functional properties of SR Ca^{2+} pump we studied the effect of the enzyme on Ca^{2+}-dependent ATP hydrolysis and on Ca^{2+} transport. The rate of Ca^{2+}-dependent ATP hydrolysis by SRVs was measured at a calculated free Ca^{2+} concentration of 10μM, (see Materials and Methods) which, in agreement with other authors [18,19], we found to represent the optimal concentration for the Ca^{2+}-dependent ATP hydrolysis. Moreover in all assays the presence of sodium azide [16 added to inhibit the mitochondrial Ca^{2+}-ATPase activity which may represent a minor contaminant in these sarcoplasmic reticulum preparations, ensured that the measured Ca^{2+}-ATPase activity was due only to SR vesicles [20]. The effect of acylphosphatase on Ca^{2+}ATPase activity is illustrated in Table 2 where a significant enhancement in the rate of ATP hydrolysis may be observed in the presence of increasing amounts of the enzyme. With 100 units/mg protein, the concentration that in the present study gave the maximal effect on the phosphate release from the EP intermediate, Ca^{2+}-ATPase activity was almost doubled with respect to basal values observed without added acylphosphatase.

The effect on calcium transport was studied by measuring the ATP-dependent Ca^{2+} influx into SRVs. These assays were performed in the same experimental conditions as those used to study the effects on ATP hydrolysis, except for the presence of oxalate which was added in order to enhance the amount of transported Ca^{2+} into the vesicles, being ATPase activity not affected by this compound [2]. Thus, oxalate-facilitated Ca^{2+}uptake was measured and expressed as nmol Ca^{2+} transported/min per mg SRV protein. Acylphosphatase significantly stimulated the rate of Ca^{2+} transport (Figure 2), and more

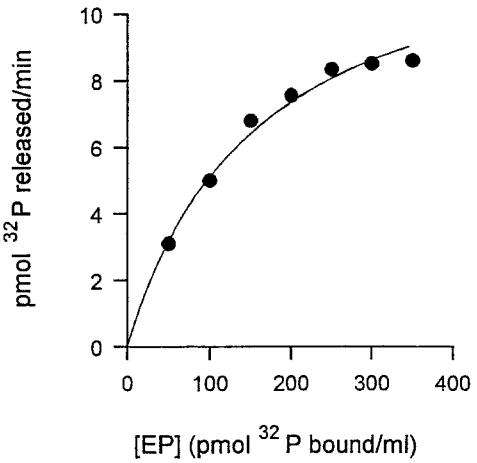

Fig.1 Initial rate of cardiac SR Ca^{2+}-ATPase intermediate dephosphorylation by acylphosphatase as a function of EP concentration.
100 units of acylphosphatase were incubated in 30mM Tris-HCl pH 7.4 at 37 °C for 30 sec with differing amounts of phosphorylated membranes. EP concentration in the medium was expressed as pmol ^{32}P bound/ml. Values, net for spontaneous hydrolysis, were calculated as described under Materials and Methods. Each point represents the mean value of five determinations

Table 2
Effect of acylphosphatase on Ca^{2+}-ATPase activity of cardiac sarcoplasmic reticulum vesicles.

Acylphosphatase (units/mg SRV protein)	Ca^{2+}-ATPase activity (nmol/min/mg SRV protein)
none	109.06 ± 5.03
25	122.62 ± 5.89
50	144.43 ± 5.78
75	175.27 ± 4.89
100	208.70 ± 4.57

The effects of acylphosphatase on Ca^{2+}-ATPase activity of cardiac SRVs was measured as described under Materials and Methods. Each value represents the mean ± S.E.M. of five determinations. Changes observed with differing amounts of acylphosphatase were statistically significant ($p<0.01$ by the one-way analysis of variance).

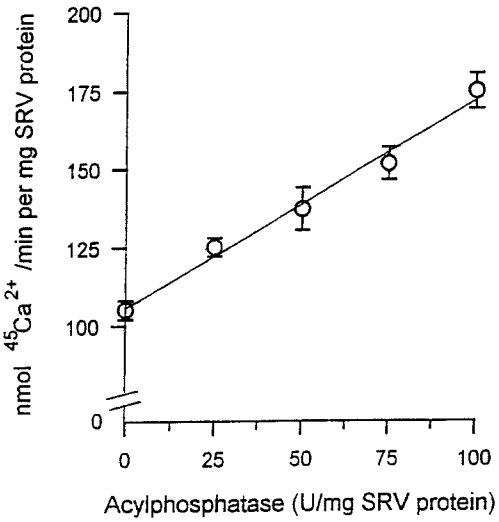

Fig.2. Effect of acylphosphatase on $^{45}Ca^{2+}$ transport into cardiac SRVs.
SRVs were incubated and assayed, as described under Materials and Methods, with varying amounts of acylphosphatase. Ca^{2+} uptake was expressed as nmol $^{45}Ca^{2+}$/min per mg SRV protein. Each point represents the mean ± S.E.M. of five determinations. Changes observed with differing amounts of acylphosphatase were statistically significant ($p<0.01$ by the one-way analysis of variance).

markedly so with higher enzyme amounts. With 100 units/mg SRV protein the enhancement of Ca^{2+}uptake was comparable to the above indicated stimulation of Ca^{2+}-dependent ATP hydrolysis .

DISCUSSION

In the present study we found that acylphosphatase actively hydrolyzes the phosphoenzyme intermediate of heart SR Ca^{2+}-ATPase. This was predictable given the acylphosphate nature of the intermediate, and all the more so that we had previously observed similar acylphosphatase effects on other transport ATPases. However, a remarkable aspect of the present study is that acylphosphatase action on EP occurs at a significant extent with enzyme amounts that fall in the physiological range; furthermore, the low Km value that we found for acylphosphatase hydrolysis (in the order of 10^{-7}M) suggests a high affinity of our enzyme towards this special substrate. In fact, the Km values found for other substrates, in particular for low molecular weight cytosolic compounds, as those reported in the Introduction, are generally higher than 10^{-4}M.

We also found that acylphosphatase induced significant modifications in the functional properties of the heart SR Ca^{2+}-ATPase, namely in the rate of both ATP hydrolysis and calcium transport. To compare these effects and to obtain a reliable estimation of the coupling between ATP hydrolysis and Ca^{2+}transport, all the experiments were conducted under the same experimental conditions as regards the temperature and the concentrations of ATP and free Ca^{2+}. In such conditions, that ensured a good efficiency of the pumping activity, the system without acylphosphatase exhibited a Ca^{2+}/ATP ratio of

0.96 near the reported value of 0.81 [21]. Since the effects of acylphosphatase on active Ca^{2+}influx into SRVs and on Ca^{2+}-dependent ATP hydrolysis were quantitatively similar (both these effects being similar to those on the EP intermediate), the Ca^{2+}/ATP ratio was not significantly modified by our enzyme addition.

A reasonable interpretation of the results here reported is that an accelerated EP turnover, induced by acylphosphatase owing to its hydrolytic effect, may result in an enhanced activity of heart SR Ca^{2+}pump, with a stimulation of both ATP hydrolysis and of active Ca^{2+}transport. Moreover, since the coupling between these two processes appears to be not modified by acylphosphatase, it may be supposed that acylphosphatase -induced hydrolysis of EP occurs without altering the normal ordered reaction sequence underlying the functioning of SR Ca^{2+}pump, in any case without hindering the conformational changes needed for Ca^{2+}transport.

In conclusion, the results obtained in this study suggest a novel mechanism that might affect the activity of heart SR Ca^{2+}-ATPase, thus contributing to the control of calcium levels in heart muscle. To judge the possibility of physiological significance of these findings, further studies are in progress in our laboratory about the details of acylphosphatase action and the relationship between the effects of our enzyme and those of other known regulators of heart SR Ca^{2+}-ATPase, namely the system centring in phospholamban interaction.

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