Stimulation of Cardiac Sarcoplasmic Reticulum Calcium Pump by Acylphosphatase

RELATIONSHIP TO PHOSPHOLAMBN PHOSPHORYLATION*

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Ca\(^{2+}\) transport by cardiac sarcoplasmic reticulum is tightly coupled with the enzymatic activity of Ca\(^{2+}\)-dependent ATPase, which forms and decomposes an intermediate phosphoenzyme. Heart sarcoplasmic reticulum Ca\(^{2+}\) pump is regulated by cAMP-dependent protein kinase (PKA) phospholamban phosphorylation, which results in a stimulation of the initial rates of Ca\(^{2+}\) transport and Ca\(^{2+}\)-ATPase activity. In the present studies we found that acylphosphatase from heart muscle, used at concentrations within the physiological range, actively hydrolyzes the phosphoenzyme of cardiac sarcoplasmic reticulum Ca\(^{2+}\) pump, with an apparent \(K_m\) on the order of 10\(^{-7}\) M, suggesting an high affinity of the enzyme for this special substrate. In unphosphorylated vesicles acylphosphatase enhanced the rate of ATP hydrolysis and Ca\(^{2+}\) uptake with a concomitant significant decrease in apparent \(K_m\) for Ca\(^{2+}\) and ATP. In vesicles whose phospholamban was PKA-phosphorylated, acylphosphatase also stimulated the rate of Ca\(^{2+}\) uptake and ATP hydrolysis but to a lesser extent, and the \(K_m\) values for Ca\(^{2+}\) and ATP were not significantly different with respect to those found in the absence of acylphosphatase. These findings suggest that acylphosphatase, owing to its hydrolytic effect, accelerates the turnover of the phosphoenzyme intermediate with the consequence of an enhanced activity of Ca\(^{2+}\) pump. It is known that phosphorylation of phospholamban results in an increase of the rate at which the phosphoenzyme is decomposed. Thus, as discussed, a competition between phospholamban and acylphosphatase effect on the phosphoenzyme might be proposed to explain why the stimulation induced by this enzyme is less marked in PKA-phosphorylated than in unphosphorylated heart vesicles.

Active Ca\(^{2+}\) transport across the membranes of sarcoplasmic reticulum (SR)\(^1\) plays a central role in the excitation-contraction coupling of cardiac muscle. More specifically, this process, in concert with the activities of two sarcolemmal systems, namely the Ca\(^{2+}\)-ATPase and the Na\(^+\)/Ca\(^{2+}\) exchanger (1), is essential to promote muscle relaxation by rapidly removing Ca\(^{2+}\) from the cytosol (2, 3). As well as in skeletal muscle, the energy-dependent calcium transport into cardiac SR depends on the activity of a Ca\(^{2+}\)-dependent ATPase (EC 3.6.1.3, ATP phosphohydrolase), which functions as a Ca\(^{2+}\)-pump, transducing chemical energy of ATP into osmotic work, consisting in a gradient of calcium ions across the SR membrane. In fact, Ca\(^{2+}\) translocations are tightly coupled with ATP hydrolysis, which is accomplished by SR Ca\(^{2+}\)-ATPase through a complex series of reactions involving the formation and the decomposition of a phosphoenzyme intermediate (3). As it occurs for other transport ATPase, the phosphoenzyme (EP) of SR Ca\(^{2+}\)-ATPase was recognized as an acylphosphatase, since phosphorylation takes place at a carboxyl group of an aspartic acid residue (4, 5). A distinctive feature of cardiac SR Ca\(^{2+}\)-ATPase is its regulation by a specific membrane protein, named phospholamban, whose phosphorylation by a cAMP- or a Ca\(^{2+}\)-calmodulin-dependent protein kinase leads to an increase in the rate of active Ca\(^{2+}\) transport (6).

Acylphosphatase (EC 3.6.1.7), a widespread enzyme that is well represented in skeletal and heart muscle (7), catalyzes the hydrolysis of the carboxylphosphate bond of acylphosphates such as 3-phosphoglyceroyl phosphate (8), carbamoyl phosphate (9), and succinyl phosphate (10). For several years we have been investigating structural and functional properties of acylphosphatase purified from muscle tissue of various animal species. More recently we have found that this enzyme, a cytosolic highly basic protein (its pl is approximately 11), in addition to the above mentioned low molecular weight soluble substrates, can hydrolyze the acylphosphorylated intermediates involved in the action mechanism of some transport ATPases, notably those of erythrocyte membrane (11) and of heart sarcolemma Ca\(^{2+}\)-ATPase (12).

In the present paper we report the results of studies that we conducted 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. Possible changes in acylphosphatase effects upon phospholamban phosphorylation were also investigated.

MATERIALS AND METHODS

Cyclic AMP-dependent protein kinase from bovine heart, 3',5'-cyclic AMP, K\(^+\)-oxalate, and Tris-ATP were from Sigma Chemie, Milano, Italy. \(^{32}\)P\(\text{ATP (300 Ci/mmol)}\) and \(^{40}\text{CaCl}_2\) (29.77 mCi/mg) were purchased from NEN DuPont (Brussels, Belgium). Nitrocellulose filters (0.45 µm) were obtained from Sartorius (Firenze, Italy). All other compounds were of analytical grade.

Acylphosphatase was purified to homogeneity from bovine heart according to Ramponi et al. (13) for the extraction and according to Stefani et al. (14) for the other steps. The enzyme, isolated as a pure product, had a specific activity of 3650 units/mg of protein using benzoyl phosphate as substrate (15). Benzoyl phosphate was synthesized as per Camici et al. (16). Cardiac sarcoplasmic reticulum vesicle (SRV) protein content was assayed by the biuret method of Beisenherz et al. (17) using...
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bovine serum albumin as a standard. Preparations of Cardiac SRVs—All operations were carried out at 4°C. Cardiac SRVs were isolated from trimmed calf ventricles according to Jones et al. (20), except that the Ca2+-oxalate loading step was omitted. The final vesicles were stored frozen at –20°C in 2 mM Hepes, pH 7.0, 0.3 M KCl, 0.3 mM sucrose. In a typical preparation, a yield of 55–65 mg of vesicle protein/100 g of wet tissue was routinely obtained. Ouabaine-sensitive Na+-K+-ATPase (see below) and cytochrome c oxidase (19) activities were measured to determine the extent of contamination of SR fraction by sarcolemma and mitochondria. Preparations of Cardiac SRVs—Phosphorylation of SRVs was carried out at 0°C according to Beekman et al. (20) with slight modifications. The standard reaction mixture (total volume 1 ml) contained 30 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 125 mM KCl, 125 mM CaCl2, or 3 mM EGTA, and 1 mg of cardiac SRVs. The reaction was started by the addition of 10 µM [γ-32P]ATP (0.2 mCi/µmol), and after 30 s of incubation the reaction was stopped by adding 4 ml of ice-cold 6% trichloroacetic acid containing 1 mM ATP and 5 mM NaH2PO4. The suspension was centrifuged at 30,000 g for 10 min, and the supernatant was discarded. Then the pellet was washed once with the above trichloroacetic acid mixture and two more times with 1 M Tris-HCl, pH 7.4. The final pellet was resuspended in 30 mM 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 32P incorporated into vesicles in the presence of CaCl2 or EGTA. Phosphorylated vesicles (1 mg/ml) were incubated in 30 mM Tris-HCl, pH 7.4, at 37°C without and with differing amounts of acylphosphatase. After 30 s, the reaction was stopped with 1 volume of ice-cold 10% trichloroacetic acid, and the suspension was centrifuged at 13,000 g for 5 min. Aliquots of the supernatant were taken to measure 32P radioactivity. The release of 32P from EP was expressed in pmol/min, since in preliminary experiments performed with variable amounts of phosphorylated vesicles we found that it proceeded linearly over 2 min. In another series of experiments, differing amounts of phosphorylated vesicles 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. To estimate the turnover rate of EP, SRVs were phosphorylated at 15°C according to Shigekawa et al. (21) in the absence and in the presence of varying amounts of acylphosphatase. Reactions were started by the addition of 20 µM [γ-32P]ATP (0.1 mCi/µmol) and terminated after 30 s by the addition of ice-cold 10% trichloroacetic acid (5% final concentration) containing 0.1 mM NaH2PO4 and 1 mM ATP. After centrifugation, aliquots were taken from the supernatant and phosphatase was found to be identical with that in Nassi et al. (22), while the pellets were treated according to the above procedure (21). Both Ca2+-dependent ATPase activity and Ca2+-independent phosphoenzyme level were estimated by subtracting the respective values observed with 1 mM EGTA from those obtained in the presence of CaCl2.

To measure the time course of undenatured phosphoenzyme decomposition, SRVs were phosphorylated at 15°C in the above-described conditions (21), 30 mM ADP, 2.1 mM [γ-32P]ATP, 145 mM EGTA (25 µl) were added to 0.5 ml of reaction medium, and the time courses of the phosphoenzyme decomposition were measured. ATPase Activity Measurements—For Ca2+-ATPase, total activity was assayed in a standard reaction mixture containing 50 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 100 mM KCl, 5 mM NaF, 50 µM CaCl2, 3 mM ATP, and 50 µg/ml vesicle protein. To determine the basal ATPase activity, the assay was carried out in presence of 1 mM Tris-EGTA instead of CaCl2. 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 ice-cold 20% trichloroacetic acid. After centrifugation (12,000 rpm for 5 min), the amount of P32 released was measured according to Baykov et al. (23) in aliquots of the supernatant. Ca2+-dependent ATPase activity was estimated by subtracting the basal ATPase activity from the total Ca2+-ATPase and was expressed as nmol/min/mg of SRV protein. Routinely, in the ATPase assays with 50 µM CaCl2 and no EGTA, a free Ca2+ concentration of approximately 10 µM was calculated using the equations of Katz et al. (24).

Ouabaine-sensitive Na+-K+-ATPase was assayed in a medium containing 50 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 100 mM NaCl, 5 mM NaN3, 1 mM Tris-EGTA, 100 mM KCl, 3 mM ATP, and 50 µg/ml vesicle protein with and without 1 mM ouabain. Ca2+ Influx Measurements into SRVs—For these measurements the reaction mixture was the same as for ATPase assays except that it included 45CaCl2 (5 μCi/µmol) 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-µm pore size), and then the filter was immediately washed twice with 4 ml of ice-cold 20 mM Tris-HCl, pH 7.4, 1 mM EGTA, and 0.1 mM HCl. Oxalate-facilitated 45Ca uptake was measured as the difference in 45Ca influx into vesicle at zero time and at the end of incubation (30 s). Radioactivity trapped on the filter was determined by liquid scintillation spectroscopy.

Treatment of SRVs with Camp-Dependent Protein Kinase (PKA) and Camp to Induce Phosphoramid Phosphorylation—SRVs (0.5 mg/ml) were incubated in 40 mM Tris-HCl, pH 7.4, 120 mM KCl, 5 mM MgCl2, 1 mM CaCl2, 10 µM AMP and 1 µg/ml PKA at 25°C for 10 min. The reaction was terminated in ice.

Aliquots were taken for assaying Ca2+ uptake and Ca2+-dependent ATPase.

In order to evidence the phosphorylation of phosphohemebian, the same phosphorylation conditions were used as described above except that 0.5 mM [γ-32P]ATP (10 µCi/ml), 25 mM Tris-EGTA, and 25 mM NaF were present according to Tada et al. (25). The reaction was stopped by the addition of a solution containing SDS, EDTA, and β-mercaptoethanol to give final concentrations of 2%, 0.1 mM, and 1%, respectively. After standing several min on ice, this mixture was incubated for 10 min at 37°C in order to solubilize the vesicles. A solution of 20 mM sodium phosphate, pH 7.2, 0.1 mM EDTA, 1% β-mercaptoethanol, 10% glycerol, and 0.005% bromphenol blue was added to the mixture, and aliquots containing 40 µg of SR protein were applied to an SDS-15% polyacrylamide gel for electrophoresis according to Laemmli (26). For autoradiography, the dried gel was exposed to Kodak X-Omat AR film with an Agfa-Gevaert (Curix MR 800) intensifier screen at –80°C for 45 min.

Data Analysis—Curves were drawn on the basis of the mean values. The data about EP dephosphorylation were analyzed by means of a linear regression analysis of observed values plotted in double reciprocal form.

The data concerning the dependence of ATP hydrolysis and Ca2+ uptake on free Ca2+ and ATP concentrations were analyzed using an equation for a general cooperative model for substrate as follows:

\[ v = \frac{V_{max} [S]^n}{K_m^o + [S]^n} \]  

(Eq. 1)

Where \( V_{max} \) (maximum velocity), \( K_{o.5} \) (concentration required to attain half-maximal velocity; apparent \( K_m \)), and \( N \) (the equivalent of the Hill coefficient) were calculated using the Fig.P computer program by Bio-soft (Cambridge, United Kingdom). Statistical analysis was performed by Student's t test or by one way analysis of variance.

RESULTS

Cardiac SRVs used for these studies were examined to determine the possibility that contaminating materials, derived from other cellular structures, were present in these preparations.

Sarcolemma and mitochondria contaminations were virtually absent since ouabain-sensitive \( \text{Na}^+ - \text{K}^+ - \text{ATPase} \) and cytochrome c oxidase activities were negligible.

Effect of Acylphosphatase on the Phosphorylated Intermediate of SRVs—SRVs incubated in presence of [γ-32P]ATP and Ca2+, as described under “Materials and Methods,” formed a Ca2+-dependent EP, whose level, after subtracting nonspecific 32P bound in presence of EGTA instead of Ca2+, was, on average, 145 pmol of 32P bound/mg of SRV protein, a value that agrees with that reported by Beekman et al. (20), from whose method our procedure derives.

To see whether the rate of EP dephosphorylation was affected by acylphosphatase, labeled vesicles were incubated with different amounts of the enzyme, from 25 to 200 units/mg of SRV protein. Such ratios were chosen because they are within the physiological range, which, in heart muscle, was estimated to be 80–130 units/mg of SRV protein (7). As shown in Fig. 1, in the presence of acylphosphatase, the release of phosphate was always higher than spontaneous hydrolysis, even at the lowest enzyme concentration, and augmented significantly with the increase in acylphosphatase/SRV protein ratio. The maximal effect was observed with 100 units/mg of SRV protein, at which concentration the phosphate release was
about 2-fold over spontaneous hydrolysis. No significant enhancement of phosphate release was observed using higher concentrations of acylphosphatase. The phosphoenzyme level was 145 pmol of $^{32}$P bound/mg of SRV protein. The initial rate of phosphate release was measured as described under "Materials and Methods" and was expressed as pmol/min. Each point is the mean ± S.E. of five experiments performed on differing vesicle preparations. All the changes in phosphate release induced by active acylphosphatase were statistically significant ($p < 0.01$ by the one-way analysis of variance). Heat-denaturated acylphosphatase (2 h at 100°C) was added at a concentration corresponding to 100 units of active enzyme/mg of SRV protein.

To evaluate the affinity of acylphosphatase toward EP, variable amounts of phosphorylated vesicles were incubated with a fixed amount of our enzyme. Acylphosphatase-induced phosphate release rose with increasing EP concentrations, and from a double reciprocal plot of these data, resulting in a straight line, an apparent $K_m$ of $157.08 ± 19.60$ nM (mean ± S.E.) was calculated (Fig. 2).

Besides these studies, which were conducted using the acid-denatured phosphoenzyme, other experiments were performed with the aim of establishing whether acylphosphatase affected the turnover rate of phosphoenzyme intermediate and had a different effect on the two EP forms, namely the ADP-sensitive (E1P) and the ADP-insensitive (E2P) form. According to Shigekawa and Akowitz (21), the rate of EP turnover was determined as the ratio between the rate of ATP hydrolysis and the phosphoenzyme level, both being measured at the steady state, which, under our conditions, was reached within 30 s after the start of the phosphorylation reaction. As shown in Fig. 3, when these measurements were taken in the presence of differing acylphosphatase amounts, the ratio increased with acylphosphatase concentrations, reaching a value that, with 100 units/mg of SRV protein, was about 2-fold greater than that in the absence of added enzyme. To explore the possibility of a different effect of acylphosphatase on E1P and E2P, we measured the time course of the phosphoenzyme decomposition after the steady state was reached and further phosphorylation was prevented by adding an excess of EGTA and MgADP. In agreement with previous reports (21) we found that phosphoenzyme decomposition exhibited an initial rapid phase without a
corresponding P_i liberation, followed by a slow phase, where the amount of P_i liberated corresponded to the decrease in EP level. The rapid phase is ascribed to the decomposition of the ADP-sensitive form of phosphoenzyme that reacts with added ADP to form ATP, whereas the slow phase represents the hydrolysis of the ADP-insensitive phosphoenzyme that does not donate its phosphate group to ADP. As it is apparent in Fig. 4, in the presence of acylphosphatase (100 units/mg of SRV protein) the rate of the slow phase of EP decomposition was markedly increased when the rate of the rapid phase was slightly affected, which suggests a preferential action of our enzyme toward the ADP-insensitive form (E2P) of phosphoenzyme.

Effect of Acylphosphatase on the Ca^{2+}-ATPase Activity and Ca^{2+} Uptake of SRVs—The rate of Ca^{2+}-dependent ATP hydrolysis by SRVs was measured at the free Ca^{2+} concentration of 10 μM, which, in agreement with other authors (27-29), we found to represent the optimal concentration for the Ca^{2+}-dependent ATP hydrolysis. As stated above, mitochondrial contamination was negligible in our SRV preparations; however, sodium azide was present in all assays to inhibit the activity of mitochondrial Ca^{2+}-ATPase, eventually present as a minor contaminant (6). This ensured that the measured Ca^{2+}-ATPase activity was only due to SRVs. In order to compare the effects on ATP hydrolysis and on Ca^{2+} transport all these determinations were performed in the same experimental conditions except that in Ca^{2+} uptake assays oxalate was added in order to enhance the amount of transported Ca^{2+} into the vesicles, since ATPase activity was not affected by this compound (3). Thus, oxalate-facilitated Ca^{2+} uptake was measured and expressed as nmol of Ca^{2+} transported/min/mg of SRV protein. A 30-s incubation was performed in Ca^{2+} transport experiments after we found that Ca^{2+} influx measured at 30-s intervals proceeded linearly over a 2 min period. As shown in Fig. 5, in the presence of increasing amounts of acylphosphatase the rates of Ca^{2+}-dependent ATP hydrolysis and of Ca^{2+} uptake were significantly stimulated. Moreover, with all the used enzyme concentrations, the enhancements of the two processes were quantitatively similar; with 100 units/SRV protein, the concentration that in the present study gave the maximal effect on the phosphate release from EP, both Ca^{2+}-ATPase activity and Ca^{2+} uptake were almost doubled with respect to the values observed without added acylphosphatase. No significant effect on these processes was observed using heat-denatured acylphosphatase.

We also studied the effect of acylphosphatase on the rate of ATP hydrolysis and of Ca^{2+} uptake as a function of free Ca^{2+} and ATP concentrations. Since a positive cooperativity was described in the Ca^{2+} dependence of calcium transport into SR, due to the presence of two Ca^{2+} binding sites in the Ca^{2+} pump (30), these data were analyzed using the Michaelis-type equation reported under "Materials and Methods," which is suitable for both cooperative and noncooperative (n = 1) behaviors. As illustrated in Fig. 6, acylphosphatase markedly increased ATP hydrolysis at all used free Ca^{2+} and ATP levels. Without the enzyme the calculated concentrations required for half-maximal ATPase activity (apparent K_m values) were 1.40 ± 0.21 μM for Ca^{2+} and 0.26 ± 0.04 mM for ATP, both findings in accordance with previous reports (28, 31). With acylphosphatase these values were significantly lower, notably 0.36 ± 0.06 μM for Ca^{2+} and 0.16 ± 0.02 mM for ATP. Both in the absence and in the presence of acylphosphatase, the calculated N values for the rate of ATP hydrolysis as a function of free Ca^{2+} and ATP concentrations were near 1, indicating the lack of positive cooperative effects.

As for the dependence of Ca^{2+} uptake on the free Ca^{2+} concentration (Fig. 7), in the absence of acylphosphatase an apparent K_m of 1.77 ± 0.23 μM and an N value of 1.42 ± 0.20 were calculated; when the enzyme was added, also in this case
at 100 units/mg of SRV protein, an increase in the rate of Ca\(^{2+}\) transport was observed at all the used free Ca\(^{2+}\) concentrations, and the apparent \(K_m\) value was significantly lowered to 1.21 ± 0.08 μM, while the value of \(N\) was not significantly changed.

**Fig. 6.** Cardiac SR Ca\(^{2+}\) ATPase activity as a function of free Ca\(^{2+}\) (A) and ATP (B) concentrations. SRVs (50 μg/ml) were assayed in the absence (E) and in the presence (●) of 100 units of acylphosphatase. Each point represents the mean value ± S.E. of five determinations.

**Fig. 7.** Cardiac SR Ca\(^{2+}\) uptake as a function of free Ca\(^{2+}\) concentration. SRVs (50 μg/ml) were assayed in the absence (E) and in the presence (●) of 100 units of acylphosphatase. Each point represents the mean value ± S.E. of five determinations.

**Fig. 8.** Autoradiogram of PKA-phosphorylated SRVs. SRVs were phosphorylated as described under "Materials and Methods" in the absence (lane 1) and in the presence (lane 2) of PKA and cAMP. Molecular mass markers are shown on left.
0.69 ± 0.09 μM for Ca\(^{2+}\) and 0.11 ± 0.02 mM for ATP, both values significantly lower than those observed in unphosphorylated vesicles (p < 0.01). As for the kinetics of Ca\(^{2+}\) transport (Fig. 10), phosphorylation resulted, as expected, in a reduction of the free Ca\(^{2+}\) concentration required for half-maximal Ca\(^{2+}\) uptake (1.17 ± 0.08 μM versus 1.77 ± 0.23 μM); in this connection, in contrast with previous reports (30) but in agreement with Movsesian (35), we did not find significant changes in the positive cooperativity for Ca\(^{2+}\) according to whether phospholamban was in its dephospho- or phospho-form. When acylphosphatase was added to the phosphorylated SRVs, always at the optimal concentration of 100 units/mg of SRV protein, the rates of both ATP hydrolysis and Ca\(^{2+}\) transport were further and significantly augmented, but the stimulatory effects of our enzyme were less striking (only about 20% over the control values) than those observed using unphosphorylated vesicles. Also the changes induced by acylphosphatase in the apparent K\(_m\) values for Ca\(^{2+}\) and ATP, both for ATPase activity and Ca\(^{2+}\) uptake, were less marked in the phosphorylated vesicles.

Table I summarizes all the observed acylphosphatase effects on the kinetic parameters of the Ca\(^{2+}\) pump ATPase in phosphorylated and unphosphorylated heart SRVs.

**DISCUSSION**

The findings here reported indicate that acylphosphatase can actively hydrolyze the phosphoenzyme intermediate of the cardiac SR Ca\(^{2+}\) pump. Such result was expected, given the acylphosphate nature of the phosphoenzyme, the catalytic properties of acylphosphatase, and our previous findings indicating a similar effect of our enzyme on the EP intermediates of other Ca\(^{2+}\)-ATPase. However, we think that two features of acylphosphatase action emerging from the present study are of interest: one is that the hydrolysis of E\(_P\) occurred to a significant extent even using an enzyme amount corresponding to the lower limit of the physiological content in heart muscle; the other is represented by the low K\(_m\) value (on the order of 10\(^{-7}\) M) that we found for EP hydrolysis, which suggests a distinctly high affinity in our enzyme toward this particular substrate, since the K\(_m\) values for other potential substrates, such as the soluble low molecular weight compounds mentioned in the Introduction, are always higher than 10\(^{-4}\) M.

When added to intact SRVs, used as a source of the Ca\(^{2+}\) pump as it exists in situ, acylphosphatase affected the functional properties of this active transport system, notably the kinetics of ATP hydrolysis and of Ca\(^{2+}\) transport. In order to compare the effects on the two processes all the experiments were performed under the same conditions of temperature, pH, and Ca\(^{2+}\) and ATP concentrations. Acylphosphatase addition, at the same concentrations used to study the effects on EP, resulted in a stimulation of the rate of ATP hydrolysis that...
matched a parallel enhancement of ATP-dependent Ca\textsuperscript{2+} influx into SRVs. Thus, although the effects were more marked with increasing acylphosphatase concentrations, no remarkable changes were observed in the stoichiometric Ca\textsuperscript{2+}/ATP ratio, which, in agreement with several previous reports (37, 38) was always near the value of 1 mol of Ca\textsuperscript{2+} transported per mol of ATP hydrolyzed. Apropos of these results, it should be noted that acylphosphatase does not exhibit per se hydrolytic activity on ATP, nor did it aid in the induction changes in the ATP-independent Ca\textsuperscript{2+} influx into SRVs (data not shown); thus, the increase in the rate of ATP hydrolysis may only be ascribed to a stimulation of the Ca\textsuperscript{2+} pump ATPase activity, while the enhancement of Ca\textsuperscript{2+} transport is not the result of a change in the passive permeability of SR membrane to this cation. It is also noteworthy that heat-denatured acylphosphatase (2 h at 100 °C), which did not affect the phosphate release from EP, also did not modify ATPase and Ca\textsuperscript{2+} pumping activities, indicating that all the observed acylphosphatase effects require the enzyme in a catalytically active form and/or in its native conformation. This also suggests a connection between the acylphosphatase effects on EP hydrolysis and on the functional properties of cardiac SR pump, all the more so that, in both cases, the modifications induced by acylphosphatase were of the same sign (stimulatory) and of the same order of magnitude.

As an interpretation of the data here reported, we propose that all the observed acylphosphatase effects are the results of an accelerated EP turnover, which, however, would not alter the normal ordered sequence of reactions and conformational transitions associated with Ca\textsuperscript{2+} transport. In other words, acylphosphatase-induced hydrolysis of EP, in competition with its own hydrolytic catalysis, would take place on the E2P form, that is at step V of the reaction system here proposed and derived from that reported by Tada et al. (3) (Fig. 11). Since E2P hydrolysis is considered to be the rate-limiting step of the entire process (21), this would result in a more rapid pumping cycle and, at the same time, could favor a shift of the equilibrium between the two proposed conformationally distinct forms of the Ca\textsuperscript{2+} pump, E\textsubscript{1} and E\textsubscript{2}, toward E\textsubscript{1}, characterized by higher affinity for Ca\textsuperscript{2+} and ATP. The results of the present study provide direct and indirect evidence in support of this interpretation. Direct evidence consists of the increased EP turnover rate that we observed in the presence of increasing acylphosphatase concentrations and in the finding that the hydrolytic effect of our enzyme was much more marked toward the ADP-insensitive form (E2P) than toward the ADP-sensitive form (E1P) of the phosphoenzyme intermediate. Indirect evidence comes from the observations that the effects of PKA phospholamban phosphorylation on the measured kinetic properties of heart SR Ca\textsuperscript{2+} pump were qualitatively and quantitatively similar to those induced by acylphosphatase. Since from the extensive studies of Tada et al. (3) phospholamban phosphorylation appears to cause the above effects enhancing both formation and decomposition of EP (39), it may be reasonable to suppose that acylphosphatase, in spite of a different kind of action, does affect in a similar way the function of the Ca\textsuperscript{2+} pump. As for the reduction in the stimulatory effect of acylphosphatase on SR Ca\textsuperscript{2+} pump upon phospholamban phosphorylation, it is difficult to explain this finding on the grounds of the data still now available. However, since it is generally accepted that unphosphorylated phospholamban inhibits the Ca\textsuperscript{2+} pump while phospholamban phosphorylation results in a relief of this inhibitory effect (40) we suggest as a tentative hypothesis that in the unphosphorylated SRVs acylphosphatase, in addition to the stimulatory effect due to its catalytic activity, could also act through another mechanism consisting of the removal of phospholamban inhibition. In fact, the basic character of acylphosphatase might favor the interaction of this enzyme protein with the Ca\textsuperscript{2+} pump, which exhibits affinity for polycationic compounds (37). Furthermore, studies from our laboratory (41) on the tridimensional structure of muscle acylphosphatase have shown that this protein contains a structural motif where 12 residues (from position 55 to 66) may be proposed to form an amphipatic α-helical structure with a prevalence of basic groups, a structure resembling that of the phospholamban cytoplasmatic 1A domain (42), which appears to be essential for the association of phospholamban with the SR Ca\textsuperscript{2+} pump. Given this structural analogy, acylphosphatase might be supposed to interact with SR Ca\textsuperscript{2+} pump, taking the place of unphosphorylated phospholamban, whose inhibitory effect would therefore be removed. However, phospholamban does not appear to be strictly necessary for acylphosphatase action, since we found (data not shown) that this enzyme had a stimulatory effect on Ca\textsuperscript{2+}-ATPase activity and on Ca\textsuperscript{2+} transport also in SRVs from fast twitch skeletal muscle (rabbit adductor magnus), which lacks phospholamban. In any case, more conclusive proofs to establish if the acylphosphatase effect on cardiac SR Ca\textsuperscript{2+} pump is due, at least in part, to a displacement of phospholamban will arise from studies in pro-

| Table 1: Effect of acylphosphatase on Ca\textsuperscript{2+}-ATPase activity and Ca\textsuperscript{2+} uptake in unphosphorylated and in PKA-phosphorylated cardiac SRVs |
|---|
| Unphosphorylated SRVs | Phosphorylated SRVs |
| Ca\textsuperscript{2+}-ATPase | Ca\textsuperscript{2+}-ATPase |
| Activity | K\textsubscript{m}Ca\textsuperscript{2+} | K\textsubscript{m}ATP | Activity | K\textsubscript{m}Ca\textsuperscript{2+} | K\textsubscript{m}ATP |
| nmol/mg min | μM | mM | nmol/mg min | μM | mM |
| Control | 109.04 ± 5.03 | 1.40 ± 0.21 | 0.26 ± 0.04 | 105.05 ± 2.31 | 1.77 ± 0.23 |
| ACPase | 208.70 ± 4.57 | 0.36 ± 0.06 | 0.16 ± 0.02 | 174.60 ± 5.81 | 1.21 ± 0.08 |

\( ^a p < 0.02 \) when compared with control values using Student's t test (n = 5).
\( ^b p < 0.05 \) when compared with control values using Student's t test (n = 7).
\( ^c \) Not significant.
gress in our laboratory; planned experiments involve the use of purified heart SR Ca\(^{2+}\)-ATPase (SERCA 2) and of a negative dominant of acylphosphatase, obtained by site-directed mutagenesis (43), which is virtually devoid of catalytic activity but retains the structural motif supposed to interact with the Ca\(^{2+}\) pump instead of phospholamban.

In conclusion, the results of the present study indicate that acylphosphatase, in its catalytically active form, can interfere with the action mechanism of heart SR Ca\(^{2+}\)-ATPase, at the same time affecting the functional properties of this active transport system. To our knowledge, this represents the first report concerning changes in the activity of heart SR Ca\(^{2+}\) pump by a cytosolic enzyme normally present in the same tissue. Certainly, at present we are not able to ascribe a physiological significance to these findings; however, from some features of acylphosphatase action (notably its high affinity for P and its ability to act even at low concentrations) such a hypothesis should be considered, in our opinion, something more than mere speculation. In any case, further studies about the details of acylphosphatase action and the molecular basis of its interaction with SR membrane would be of interest to ascertain if this enzyme may be involved in vivo, in addition to the other mechanisms now recognized, in the regulation of SR Ca\(^{2+}\) pump activity, hence in the control of calcium homeostasis in heart muscle.

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