Alteration of Acylphosphate Formation of Cardiac Sarcoplasmic Reticulum ATPase by Calmodulin-Dependent Phosphorylation

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The calcium-dependent acylphosphate formed by the calcium transport ATPase of cardiac sarcoplasmic reticulum and the calcium-, calmodulin-dependent phosphoester(s) of sarcoplasmic reticulum fractions formed by a calcium-, calmodulin-dependent membrane-bound protein kinase can be distinguished by removal of calcium and/or magnesium by EDTA or hydroxylamine treatment of the acid denaturated membranes. Both procedures decompose the acylphosphate with little effect on the phosphoester(s).

Calmodulin-dependent phosphorylation (2.44 nmol/mg SR protein) reduces the apparent K(Ca) of the acylphosphate steady state level of the calcium transport ATPase from 0.56 to 0.34 μM free calcium, without affecting the maximum phosphoenzyme level (0.93 *versus* 0.89 nmol/mg protein), and has little, if any, effect on the Hill-coefficient (1.32 *versus* 1.54).

Introduction

Calmodulin increases the rate of calcium transport [1–8] and the rate of calcium-activated ATP hydrolysis [4, 6, 7] by cardiac sarcoplasmic reticulum. The stimulation of calcium transport by calmodulin was suggested to be due to calmodulin-dependent phosphorylation of a protein with an apparent M_r of 11 kDa by a membrane-bound, calcium-, calmodulin-dependent protein kinase [2], probably a subunit of phospholamban [2, 9], which on phosphorylation by the cAMP-dependent protein kinase was shown to mediate an elevation of the rate of calcium transport by cardiac sarcoplasmic reticulum [9].

It was demonstrated recently that the calmodulin-dependent increase in the rate of calcium transport of cardiac sarcoplasmic reticulum correlated with the phosphoester formation of a 9–11 kDa protein by a calcium-, calmodulin-regulated protein kinase [8], which is at least partially due to an increase in the apparent calcium affinity of the high-affinity calcium binding sites of the transport ATPase, as judged from the shift in the calcium dependence of calcium transport [3, 8] and calcium-activated ATP splitting [6] to lower free calcium concentrations.

Abbreviations: SR, sarcoplasmic reticulum; CaM, calmodulin; EGTA (ethylene glycol bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid; PEP, phosphoenol-pyruvate; PK, pyruvate kinase.

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The present communication reports that calmodulin-dependent phosphoester formation alters the calcium dependence of the acylphosphate steady state level of the calcium transport ATPase.

Materials

Calmodulin and EGTA were purchased from Fluka AG (Buchs); ATP from Sigma Chemical Co. (St. Louis); phosphoenolpyruvate and pyruvate kinase from Boehringer GmbH (Mannheim); *ortho*[^{32}P]phosphate from New England Nuclear (Boston); all other chemicals were from E. Merck (Darmstadt).

Methods

[γ-^{32}P]ATP was prepared according to Glynn and Chappel [10]. Cardiac sarcoplasmic reticulum fractions were isolated from mongrel dogs [11] and stored at −40 °C in a medium containing 10 mM histidine buffer (pH 7.0) and 0.3 M sucrose.

Protein was measured by the Folin method [12] standardized against bovine serum albumine.

Phosphorylation of the transport ATPase from [^{32}P]ATP and calmodulin-dependent phosphorylation were carried out as described previously [7, 11] and stopped either by addition of 10–20 mM EDTA ("EDTA-stop") prior to the addition of 2–3 volumes of an acid solution containing 0.5 M perchloric acid and 0.1 M phosphoric acid or by addition of the acid solution ("acid-stop"). The protein

was recovered by centrifugation, washed once with the above acid solution, transferred to a glass fibre filter, washed again with 25 ml acid solution, finally dissolved in 2 ml cellosolve plus 8 ml atomlight and the incorporated [^{32}P]phosphate counted in a liquid scintillation counter.

Hydroxylamine treatment of acid-denaturated sarcoplasmic reticulum membranes was carried out to remove the acylphosphate of the ATPase [11, 13, 14]. The conditions were 30 min at 25 °C, pH 5.2, with 0.2 M sodium acetate buffer and 0.8 M hydroxylamine [11].

Free calcium was calculated as described previously [8] taking calcium, magnesium and potassium complexes with EGTA, ATP and phosphoenolpyruvate into consideration.

Results

Fig. 1 shows the phosphate incorporation from [^{32}P]ATP into sarcoplasmic reticulum fractions in the absence or presence of brain calmodulin and its decomposition by addition of EDTA for 5–60 s before the addition of perchloric acid plus phosphoric acid. In the absence of calmodulin the phosphoprotein declines rapidly after addition of EDTA, nearly approaching the level of phosphoprotein formed in the absence of free calcium (< 0.01 µM) but in the presence of magnesium indicating that EDTA treatment reduces the phosphoenzyme steady state level of the ATPase by about 90% within 15–30 s. The decline of phosphate incorporated in the presence of exogenous calmodulin, *i.e.* in calmodulin-dependent phosphorylation plus ATPase phosphorylation, shows a similar time course following addition of EDTA to the controls. The amount of phosphoprotein which is removed by EDTA treatment under these conditions was nearly identical with the amount which decayed in the controls, indicating that EDTA treatment removes the acylphosphate of the ATPase with little effect on the phosphoester formed in calmodulin-dependent phosphorylation (Fig. 1).

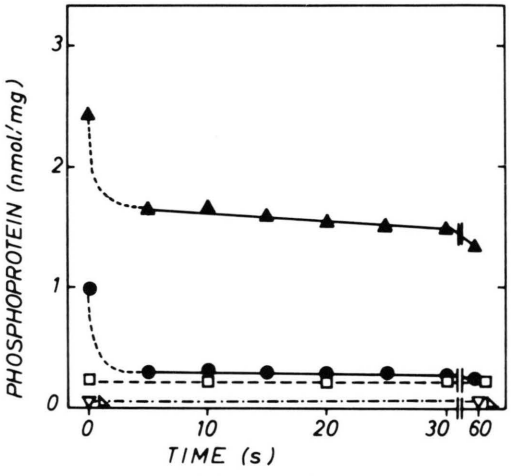

Fig. 1. Effect of EDTA on ATPase phosphoenzyme and CaM-dependent phosphorylation of cardiac SR. Phosphorylation was performed at 25 °C, pH 7.0 for 30 s in a medium containing 40 mM histidine-buffer, 5 mM azide, 2 mM PEP, 50 µg PK/ml, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM EGTA (~ 10 µM free Ca^{2+}), 0.4 mg SR/ml without (●) or with (▲) 0.1 µM CaM. Zero Ca: as above but 1 mM EGTA without added Ca (□), (control). Zero Ca, zero Mg: as above but 1 EGTA plus 1 EDTA without added Ca and Mg (▽, control; ▵, CaM). The reaction was stopped either with acid or by addition of EDTA (25 mM final concentration) for 5–60 s before the addition of the acid solution (see Methods).

The effects of EDTA treatment of native SR membranes and of hydroxylamine treatment of acid denaturated SR membranes on the acylphosphate formed by the ATPase are compared in Table I. CaM-dependent phosphorylation varied less than 5% and ATPase phosphorylation about 10%, calculated from the EDTA stop and/or hydroxylamine treatment. A small portion of incorporated phosphate remained above the level obtained in the absence of calcium but presence of magnesium with both treatments.

Fig. 2 shows the calcium dependence of the phosphoprotein steady state level of the transport ATPase of sarcoplasmic reticulum formed from [^{32}P]ATP in the absence and presence of calmodulin-dependent phosphorylation. Calmodulin-dependent phosphorylation of sarcoplasmic reticulum was carried out for 3 min prior to phosphoenzyme formation under similar condition as given in Table 1, but without an ATP regenerating system and a higher SR level of 2 mg/ml. Calmodulin-dependent phosphate incorporation was 2.44 nmol/mg SR protein ($n = 5$) determined under identical conditions with [^{32}P]ATP in parallel experiments. Phosphate incorporation into the ATPase protein was performed for 10 s ("acid stop") and the phosphate incorporation then determined with or without hydroxylamine treatment. The acylphosphate was calculated by subtracting the incorporated [^{32}P]phosphate obtained after hydroxyalmine treatment from the total incorporated phosphate observed in the absence of hydroxylamine treatment.

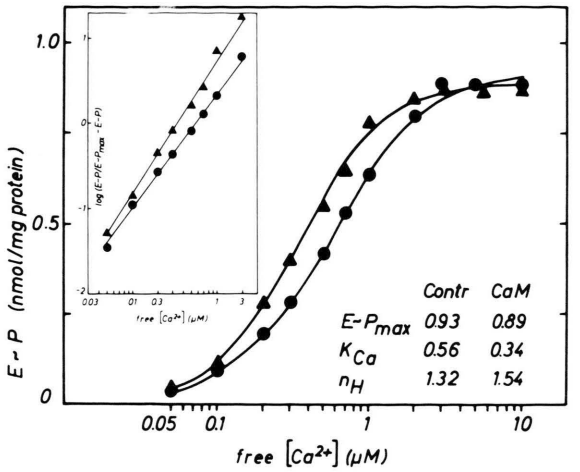

Fig. 2. Effect of CaM-dependent phosphorylation on the phosphoprotein steady state level of the Ca-ATPase of cardiac SR. CaM-dependent prephosphorylation was performed at 25 °C, pH 7.0, $\mu = 0.1$ M for 3 min in a medium containing 40 mM histidine-buffer, 5 mM azide, 37 mM KCl, 5.06 mM $MgCl_2$, 4.34 mM ATP or [^{32}P]ATP, 0.5 mM EGTA, 0.5 mM $CaCl_2$, 2 mg SR/ml, without (●) or with (▲) 0.1 μM CaM. ATPase phosphorylation was performed at 25 °C, pH 7.0, $\mu = 0.1$ M for 10 s in a medium containing 40 mM histidine-buffer, 5 mM azide, 40 mM KCl, 2 mM PEP, 40–80 μg PK/ml, 1.08 mM [^{32}P]ATP, 2.24 mM $MgCl_2$, 2 mM EGTA, 0.38–1.97 mM $CaCl_2$ (0.05–10 μM free Ca^{2+}) 0.2 mg preincubated SR/ml (1 mM free Mg^{2+}, 1 mM Mg-ATP). Zero Ca or zero Ca and zero Mg as in Fig. 1. Inset: Hill plot. Values are means of 2 experiments with different SR preparations. The theoretical curves were calculated by fitting the Hill equation to the data by an iterative, non-linear least squares approximation [8].

Table I. Comparison of "acid-stop" and "EDTA-stop" on CaM-dependent phosphorylation of cardiac SR. Ca-dependent (~ 10 μM free Ca) and Ca-independent (without added Ca, but 1 mM EGTA) phosphorylation was performed at 25 °C, pH 7.0, $\mu = 0.1$ M in 40 mM histidine-buffer, 5 mM azide, 30 mM KCl, 2 mM PEP, 40 μg/ml PK, 5.26 mM $MgCl_2$, 4.33 mM [^{32}P]ATP, 0.5 mM EGTA, 0.51 mM $CaCl_2$, 0.25 mg SR/ml for 3 min in the absence or presence of 0.1 μM CaM (1 mM free Mg^{2+}, 4 mM Mg-ATP). EDTA-stop and hydroxylamine treatment are described in the Methods. Values are means of 2 experiments with different SR preparations and corrected for unspecific phosphate incorporation in the absence of Ca and Mg.

	"Acid-stop" Control	"Acid-stop" CaM	"EDTA-stop" Control	"EDTA-stop" CaM
	[nmol/mg]		[nmol/mg]	
– Hydroxylamine				
+ Ca, + Mg	1.59	3.92	0.63	2.73
0 Ca, + Mg	0.51	0.52	0.47	0.44
+ Hydroxylamine				
+ Ca, + Mg	0.53	2.77	–	–
0 Ca, + Mg	0.39	0.40	–	–

These former values were fairly constant over the range of free calcium from 0.1–10 μM averaging 0.062 and 0.196 nmol/mg SR in control and calmodulin-dependent phosphorylation, respectively. The small difference in the retained phosphate after hydroxylamine treatment between control and calmodulin-dependent prephosphorylated membranes might be due to calmodulin-dependent phosphorylation following preincubation of the vesicles in the presence of higher calcium and calmodulin concentrations [7], possibly as a result of slow dissociation of the activating $CaM(Ca_4)$ species from the protein kinase.

The experiments demonstrate that calmodulin-dependent phosphorylation of sarcoplasmic reticulum shifts the calcium activation of phosphoenzyme steady state level to lower free calcium concentrations (apparent K(Ca): 0.56 *vs.* 0.34 μM in control and calmodulin-dependent phosphorylation, respectively). The maximum phosphoprotein (0.93 and 0.89 nmol/mg) and the Hill coefficients (1.32 and 1.54) remained unaltered (Fig. 2).

Discussion

Phosphorylation of the calcium transport ATPase of sarcoplasmic reticulum from ATP represents acylphosphate formation [13] due to phosphate incorporation into a β-aspartyl residue as revealed from borohydride reduction of acid-denaturated SR membranes [15] and by NMR studies of the phosphoprotein steady state level in native membranes [16], as has been suggested earlier from the decomposition of the acid-denaturated phosphoenzyme by hydroxylamine [11, 13, 14]. Calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum by the calcium-, calmodulin regulated protein kinase represents serin phosphoester formation [17] insensitive to hydroxylamine [2, 5–8], which occurs to more than 90% into a 9–11 kDa protein as revealed from polyacrylamide gel electrophoresis of phosphorylated sarcoplasmic reticulum membranes solubilized in 2% sodium dodecyl sulfate at 100 °C [7, 8; s. ref. 2, 5, 6].

The present communication demonstrates that calmodulin-dependent phosphorylation of sarcoplasmic reticulum membranes affects the phosphoprotein steady state level of the transport ATPase measured at conditions which exclude any contribution by calmodulin-dependent phosphoester towards

the increase in acylphosphate level. The lowering of the apparent K(Ca) in the presence of calmodulin-dependent phosphorylation indicates an increase in apparent calcium affinity of the high-affinity calcium binding sites of the ATPase and is in good agreement with the reduction of the apparent K(Ca) of the rate of calcium transport [3, 8] and rate of calcium-activated ATP-hydrolysis [6]. The present results are at variance with the findings of Lopaschuk *et al.* [4], who reported that calmodulin reduces the phosphoprotein steady state level of the cardiac calcium transport ATPase.

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