Biochimica et Biophysica Acta, 732 (1983) 99–109
Elsevier

BBA 71714

CALMODULIN-DEPENDENT ELEVATION OF CALCIUM TRANSPORT ASSOCIATED WITH CALMODULIN-DEPENDENT PHOSPHORYLATION IN CARDIAC SARCOPLASMIC RETICULUM

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(Received January 21st, 1983)

Key words: Ca^{2+} transport; ATPase; Calmodulin; Phosphorylation; (Cardiac sarcoplasmic reticulum)

The rate of calcium transport by sarcoplasmic reticulum vesicles from dog heart assayed at 25°C, pH 7.0, in the presence of oxalate and a low free Ca^{2+} concentration (approx. 0.5 μM) was increased from 0.091 to 0.162 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ with 100 nM calmodulin, when the calcium-, calmodulin-dependent phosphorylation was carried out prior to the determination of calcium uptake in the presence of a higher concentration of free Ca^{2+} (preincubation with magnesium, ATP and 100 μM $CaCl_2$; approx. 75 μM free Ca^{2+}). Half-maximal activation of calcium uptake occurs under these conditions at 10–20 nM calmodulin. The rate of calcium-activated ATP hydrolysis by the Ca^{2+}-, Mg^{2+}-dependent transport ATPase of sarcoplasmic reticulum was increased by 100 nM calmodulin in parallel with the increase in calcium transport; calcium-independent ATP splitting was unaffected. The calcium-, calmodulin-dependent phosphorylation of sarcoplasmic reticulum, preincubated with approx. 75 μM Ca^{2+} and assayed at approx. 10 μM Ca^{2+} approaches maximally 3 nmol/mg protein, with a half-maximal activation at about 8 nM calmodulin; it is abolished by 0.5 mM trifluperazine **. More than 90% of the incorporated [^{32}P]phosphate is confined to a 9–11 kDa protein, which is also phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase and most probably represents a subunit of phospholamban. The stimulatory effect of 100 nM calmodulin on the rate of calcium uptake assayed at 0.5 μM Ca^{2+} was smaller following preincubation of sarcoplasmic reticulum vesicles with calmodulin in the presence of approx. 75 μM Ca^{2+}, but in the absence of ATP, and was associated with a significant degree of calmodulin-dependent phosphorylation. However, the stimulatory effect on calcium uptake and that on calmodulin-dependent phosphorylation were both absent after preincubation with calmodulin, without calcium and ATP, suggestive of a causal relationship between these processes.

Introduction

It has been demonstrated for cardiac sarcoplasmic reticulum that phosphorylation of a 22–24 kDa protein [1–6] designated phospholamban [1,2], by a cAMP-dependent protein kinase gives rise to an increase in the rate of calcium transport and in the rate of calcium-activated ATP hydrolysis [1–7]. The stimulation of the rate of calcium transport of cardiac sarcoplasmic reticulum by the calcium-binding protein calmodulin [7–10] was suggested to be mediated, too, by phosphorylation of phospholamban by a calcium-, calmodulin-dependent protein kinase [9], but the correlation between

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** 10-[3-(4-Methylpiperazin-1-yl)propyl]-2-trifluoromethylphenothiazine; trifluoperazine.

Abbreviation: Mops, morpholinopropanesulfonic acid.

these two processes has not yet been unequivocally proven. The association of calmodulin-dependent phosphorylation with stimulation of calcium transport and calcium-dependent ATP splitting by cardiac sarcoplasmic reticulum has quite recently been demonstrated with high concentrations of calmodulin in the micromolar range [12]. The present study describes stimulation of calcium transport and calcium-dependent ATP hydrolysis by cardiac sarcoplasmic reticulum with low concentrations of calmodulin in the presence of calmodulin-dependent phosphorylation, but not in its absence.

Materials and Methods

Reagents

Carrier-free [^{32}P]orthophosphoric acid was purchased from New England Nuclear (Boston); $^{45}CaCl_2$ from Amersham International (Amersham); ATP, the catalytic subunit of cAMP-dependent protein kinase (from bovine heart), the cAMP-dependent protein kinase (from rabbit skeletal muscle) from Sigma (St. Louis); phospho*enol*pyruvate, pyruvate kinase, cAMP from Boehringer (Mannheim); ethylene glycol bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) and calmodulin (from bovine brain) from Fluka AG (Buchs); sodium dodecyl sulfate, acrylamide, *N,N'*-methylenebisacrylamide, from Bio-Rad (Richmond). Protein standards for estimating molecular weights of sarcoplasmic reticulum proteins were obtained from the following suppliers: phosphorylase *b*, albumin, catalase, ovalbumin, lactate dehydrogenase, carbonic anhydrase, trypsin inhibitor, ferritin, α-lactalbumin (Pharmacia, Uppsala); myoglobin and myoglobins I, II, III (Fluka AG, Buchs), aprotinin (Bayer, Leverkusen), glucagon (Eli Lilly, Indianapolis), glutamic dehydrogenase and α-chymotrypsinogen (Sigma, St. Louis). All other chemicals (analytical grade) were purchased from E. Merck (Darmstadt). [γ-^{32}P]ATP was prepared according to Glynn and Chappell [13].

Preparation of sarcoplasmic reticulum

Cardiac sarcoplasmic reticulum was isolated from dogs [14] 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 [15] standardized against bovine serum albumin.

Assays

Calcium uptake. Uptake of calcium by sarcoplasmic reticulum vesicles was measured with $^{45}CaCl_2$ [16,17] at 25°C, pH 7.0, in the absence or presence of various calmodulin concentrations in a medium containing 40 mM histidine-HCl-Tris, 2 mM phospho*enol*pyruvate, 20 μg/ml pyruvate kinase, 5 mM NaN_3, 5 mM potassium oxalate, 3.5 mM $MgCl_2$, 1 mM ATP and 0.2 mM $^{45}CaCl_2$ plus 0.3 mM EGTA ('low free Ca^{2+}' approx. 0.5 μM) or 0.2 mM $^{45}CaCl_2$ plus 0.2 mM EGTA ('high free Ca^{2+}' approx. 10 μM); the reaction was started with preincubated sarcoplasmic reticulum protein (final concentration 0.05 mg/ml). Preincubation of sarcoplasmic reticulum vesicles was carried out at 25°C, pH 7.0 for 2–5 min in the absence or presence of calmodulin in a medium containing 40 mM histidine-HCl-Tris, 2 mM phospho*enol*pyruvate, 20 μg/ml pyruvate kinase, 5 mM NaN_3, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM $CaCl_2$ (approx. 75 μM free Ca^{2+}) and 0.5 mg sarcoplasmic reticulum protein/ml (if not otherwise stated in the legends) i.e., with unlabeled $CaCl_2$ and in the absence of oxalate. The calmodulin concentrations (5–300 nM) in the preincubation and uptake media were always identical.

0.5 ml of the uptake medium was filtered through a cellulose filter of 0.3 μm pore size (Schleicher and Schüll; Selectron Type BA84) prewashed with 1 ml 100 mM sucrose prior to filtration. The protein on the filter was washed with 3 ml of a solution containing 100 mM sucrose plus 1 mM Tris-EGTA (pH 7.0). The radioactivity on the filter was counted in a Liquid Scintillation Counter in 2 ml cellosolve plus 8 ml atomlight (New England Nuclear, Boston) as scintillator.

Calcium uptake was also measured spectrophotometrically with the aid of Arsenazo III [18] using a dual-wavelength spectrophotometer (Sigma ZWS II) equipped with a stopped-flow apparatus. Sarcoplasmic reticulum vesicles (0.85 mg/ml) were preincubated at 25°C for 3 min in 40 mM Mops-Tris (pH 7.0), 5 mM NaN_3, 5 mM phospho*enol*-pyruvate, 50 μg/ml pyruvate kinase, 1 mM $MgCl_2$,

1 mM ATP, 0.1 mM $CaCl_2$, with or without 200 nM calmodulin.

Calcium uptake was measured at 25°C with 40 mM Mops-Tris (pH 7.0), 4 mM NaN_3, 5 mM phospho*enol*pyruvate, 50 μg/ml pyruvate kinase, 5 mM $MgCl_2$, 1 mM ATP, 5 mM potassium oxalate, 0.01 mM Arsenazo III, 200 nM calmodulin in syringes I and II, 0.34 mg preincubated sarcoplasmic reticulum protein/ml and 0.02 mM $CaCl_2$ in syringe I and 0.08 mM $CaCl_2$ in syringe II. The wavelength pairs of 650 and 685 nm were used to record the increase in absorbance [18].

ATPase. Total ATPase of sarcoplasmic reticulum vesicles was measured with [γ-^{32}P]ATP as substrate at 25°C (pH 7.0) in the presence or absence of 100 nM calmodulin in a medium containing 40 mM histidine-HCl-Tris, 2 mM phospho*enol*pyruvate, 20 μg/ml pyruvate kinase, 5 mM NaN_3, 5 mM potassium oxalate, 3.5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 0.2 mM $CaCl_2$, 0.3 mM EGTA (approx. 0.5 μM free Ca^{2+}); the reaction was started with preincubated sarcoplasmic reticulum protein (final concentration 0.05 mg/ml). The calcium-independent and magnesium-dependent ATPase activity was measured under identical conditions except that no calcium was added and the EGTA concentration was 1 mM. The calcium-, magnesium-dependent ATP splitting of the transport ATPase was calculated by subtracting the magnesium-dependent ATPase activity from the total ATPase activity [16]. Preincubation of sarcoplasmic reticulum vesicles in the presence or absence of 100 nM calmodulin was carried out at 25°C (pH 7.0) for 2 min in a medium containing 40 mM histidine-HCl-Tris, 2 mM phospho*enol*pyruvate, 20 μg/ml pyruvate kinase, 5 mM NaN_3, 1 mM $MgCl_2$, 1 mM unlabeled ATP, 0.1 mM $CaCl_2$ and 0.5 mg sarcoplasmic reticulum protein/ml. The reaction was stopped with perchloric acid (final concentration 5%, v/v). The [^{32}P]orthophosphate liberated from [γ-^{32}P]ATP was measured by extraction with isobutane/benzene/molybdate [19].

Phosphorylation. Phosphorylation of the calcium transport ATPase of sarcoplasmic reticulum [14] and calcium-, calmodulin-dependent phosphorylation of sarcoplasmic reticulum proteins by [γ-^{32}P]ATP was carried out at 25°C, pH 7.0, in a medium containing 40–60 mM histidine-HCl-Tris, 5 mM NaN_3, 3.5–10 mM $MgCl_2$, 1–4 mM [γ-^{32}P]ATP, 0.33 mM $CaCl_2$ plus 0.5 mM EGTA (approx. 0.5 μM free Ca^{2+}) or 0.5 mM $CaCl_2$ plus 0.5 mM EGTA (approx. 10 μM free Ca^{2+}), 0.16–0.4 mg sarcoplasmic reticulum protein/ml, with or without 2 mM phospho*enol*pyruvate and 40 μg/ml pyruvate kinase, and in the absence or presence of 2–300 nM calmodulin. Both types of phosphorylation were carried out with or without preincubation of sarcoplasmic reticulum protein as detailed in the legends. Calcium-independent, magnesium-dependent phosphorylation of sarcoplasmic reticulum fractions from [γ-^{32}P]ATP were performed similarly, but without addition of calcium and in the presence of 1 mM EGTA plus 5 mM $MgCl_2$.

The phosphorylation reaction was stopped by addition of 2–4 vol. ice-cold solution containing 0.5 M perchloric acid and 100 mM phosphoric acid. The protein was recovered by centrifugation and washed once with 10 ml of the above solution and then transferred to a glass-fiber filter (Gelman Sciences Inc., Type A/E) and washed again with 25 ml 0.5 M perchloric acid/100 mM phosphoric acid [20]. The protein was dissolved in 2 ml cellosolve plus 8 ml atomlight and the radioactivity was counted in a liquid scintillation counter.

Hydroxylamine treatment of acid-denaturated phosphoproteins of sarcoplasmic reticulum fractions [14] was carried out after three washes of the phosphorylated proteins with the stopping solution, one wash with 0.5 M perchloric acid, one wash with 0.1 M perchloric acid plus 0.2 M NaCl and two washes with distilled water, followed by resuspension of the protein in 0.2 M sodium acetate buffer (pH 5.2)/0.4 M hydroxylamine (25°C; 20 or 60 min); the reaction was stopped by addition of 0.5 M perchloric acid/100 mM phosphoric acid and the protein recovered by centrifugation, followed by two washes with the above medium, or two washes with distilled water when the protein was used for slab gel electrophoresis.

The changes in specific activity of [γ-^{32}P]ATP during phosphorylation due to the use of the ATP regenerative system did not exceed 5% under the present conditions and were neglected in the calculations of the phosphoprotein values.

Activation of calmodulin-deficient phosphodiesterase by calmodulin [21,22] was per-

formed in a medium containing 40 mM Tris-HCl (pH 8.0), 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$, 0.2 U/ml 5'-nucleotidase, 0.026 U/ml calmodulin-deficient phosphodiesterase, 2 mM cAMP, 0.05–100 nM calmodulin, or 0.5–200 µl/ml of supernatant of boiled sarcoplasmic reticulum fractions (30°C; 30 min).

Polyacrylamide gel electrophoresis

Electrophoresis of phosphorylated proteins of the sarcoplasmic reticulum fractions was performed according to Laemmli [23], with minor modifications. Linear gradient slab gels (7–20%, acrylamide/ *N,N'*-methylenebisacrylamide, 30/0.8; medium: 0.375 M Tris-HCl (pH 8.8)/0.1% SDS/0.075% ammonium persulfate/0.1% tetramethylenediamine; 10 × 14 × 0.75 cm) and a stacking gel (3%; acrylamide/*N,N'*-methylenebisacrylamide, 30/0.8; medium 0.125 M Tris-HCl (pH 6.8)/0.1% SDS/0.15% ammonium persulfate/0.1% tetramethylenediamine; 3 × 14 × 0.75 cm) were used. The electrode buffer was 0.105 M Tris base (pH 8.8)/0.192 M glycine/0.1% SDS/1 mM EDTA. 20 µg solubilized protein (in a solution comprising 0.05 M Tris-HCl (pH 6.8)/2% SDS/5% mercaptoethanol/10% glycerol/0.001% Bromophenol blue, either incubated at 37°C for 20 min or treated in boiling water for 2 min) were applied to each well. Electrophoresis was performed at room temperature (22–24°C) at 60 V (stacking gel) and 140 V (separating gel) for about 4.5 h. Gels were stained in 0.25% Coomassie blue/7.5% acetic acid/5% methanol for 2–3 h and destained by diffusion in 7% acetic acid/5% methanol, dried on a filter paper under vacuum. Autoradiography was undertaken on a Kodak OG1 film for 24 h.

Calculation

Calculation of free Ca^{2+} concentrations in calcium- and EGTA-containing solutions was performed as described previously [20] using the association constants (log K_a) of 11 and 5.3 for the $Ca \cdot EGTA^{2-}$ and $Ca \cdot HEGTA^{-}$ complexes given by Schwarzenbach [24] for a temperature of 20°C and 0.1 M ionic strength.

Results

Calmodulin-dependent stimulation of calcium uptake and ATPase by sarcoplasmic reticulum

Fig. 1 shows the effect of calmodulin on the rate of calcium uptake by sarcoplasmic reticulum vesicles investigated at 25°C, pH 7.0, and a low concentration of free calcium of about 0.5 µM. The rate of calcium uptake measured with $^{45}CaCl_2$ following preincubation of sarcoplasmic reticulum vesicles with 100 nM calmodulin in the presence of 0.1 mM unlabelled $CaCl_2$, 1 mM $MgCl_2$ and 1 mM ATP for 2–5 min is markedly greater than the rates obtained with control vesicles preincubated under identical conditions in the absence of calmodulin. Preincubation of sarcoplasmic reticulum vesicles with calmodulin in the presence of 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, but without ATP, results in a delayed and smaller increase in the rate of calcium uptake during incubation with $^{45}CaCl_2$ (Fig. 1, Table II). Preincubation of sarcoplasmic reticulum vesicles with calmodulin in the absence of $CaCl_2$, $MgCl_2$ and ATP does not significantly

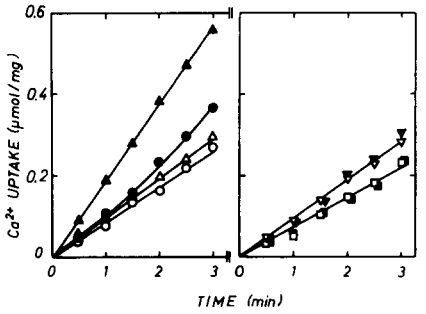

Fig. 1. Effect of calmodulin on calcium uptake by sarcoplasmic reticulum vesicles at low free $^{45}Ca^{2+}$. Sarcoplasmic reticulum vesicles were preincubated for 2 or 5 min at 25°C without (△, ○, ▽, □) or with 100 nM calmodulin (▲, ●, ▼, ■) in the presence of 40 mM histidine-HCl-Tris (pH 7.0), 2 mM phospho*enol*pyruvate, 20 µg/ml pyruvate kinase, 5 mM NaN_3 (▽, ▼); or plus 0.1 mM $CaCl_2$ (approx. 75 µM free Ca^{2+}), 1 mM $MgCl_2$, 1 mM ATP (△, ▲); or plus 0.1 mM $CaCl_2$, 1 mM $MgCl_2$ (○, ●); or plus 0.05–0.1 mM EGTA, 1 mM $MgCl_2$, 1 mM ATP (□, ■). Calcium uptake was measured at 25°C in a medium containing 40 mM histidine-HCl-Tris (pH 7.0), 2 mM phospho*enol*pyruvate, 20 µg/ml pyruvate kinase, 5 mM NaN_3, 5 mM potassium oxalate, 3.5 mM $MgCl_2$, 1 mM ATP, 0.2 mM $^{45}CaCl_2$, 0.3 mM EGTA (approx. 0.5 µM free Ca^{2+}), 0.05 mg/ml preincubated sarcoplasmic reticulum protein with or without 100 nM calmodulin. Values are means from two experiments with different sarcoplasmic reticulum preparations.

affect the rate of calcium uptake. Preincubation of sarcoplasmic reticulum vesicles with or without calmodulin in the presence of 1 mM $MgCl_2$ and 1 mM ATP, but with 0.05–0.1 mM EGTA instead of $CaCl_2$ shows that 100 nM calmodulin has no effect on the rate of calcium uptake. The actual rate of calcium uptake is reduced by about 20% as compared with the rate of calcium uptake in the absence of EGTA during the preincubation period.

Fig. 2 shows the effect of various calmodulin concentrations on the rate of calcium uptake by sarcoplasmic reticulum vesicles assayed at approx. 0.5 μM free $^{45}Ca^{2+}$ following preincubation with 0.1 mM unlabelled calcium, magnesium, ATP and calmodulin. Half-maximal activation of calcium transport occurs at 10–20 nM calmodulin.

Fig. 3 shows the effect of brain calmodulin and also of calmodulin present in the heart sarcoplasmic reticulum preparations on calmodulin-deficient phosphodiesterase and calcium uptake by sarcoplasmic reticulum vesicles. The supernatant of boiled, pooled sarcoplasmic reticulum fractions, which was dialysed and concentrated by lyophylisation, activates the calmodulin-deficient phosphodiesterase in an identical manner to brain calmodulin, indicating that the activation of the former is due to calmodulin. This conclusion is further strengthened by the fact that the supernatant of boiled sarcoplasmic reticulum fractions activates calcium uptake by sarcoplasmic reticulum vesicles (Fig. 3B). Phosphodiesterase is activated by other calcium-binding proteins such as troponin [25], but there is no indication that troponin can substitute for calmodulin in the stimulation of calcium transport by cardiac sarcoplasmic reticulum.

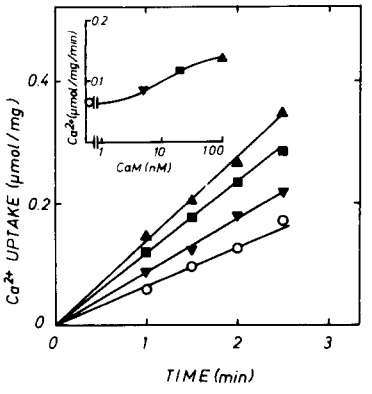

Fig. 2. Dependence of the rate of calcium uptake by sarcoplasmic reticulum vesicles on calmodulin concentrations. Preincubation of sarcoplasmic reticulum vesicles and calcium uptake with $^{45}CaCl_2$ (approx. 0.5 μM free Ca^{2+}) was performed as described in Materials and Methods. Control (○), 5 (▼), 20 (■) and 100 nM (▲) calmodulin.

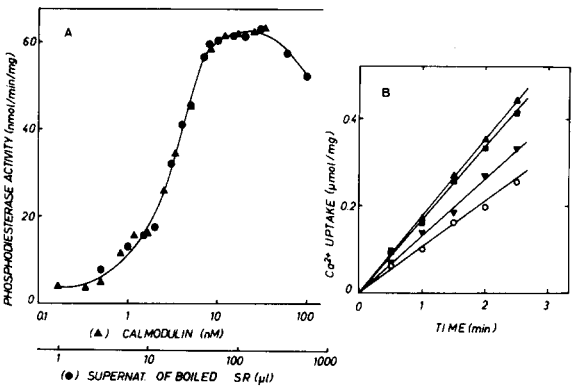

Fig. 3. Activation of calmodulin-deficient phosphodiesterase by brain calmodulin or calmodulin in isolated dog heart sarcoplasmic reticulum fractions. (A) Phosphodiesterase activation by brain calmodulin (▲) or supernatant of boiled sarcoplasmic reticulum (SR) fractions (●). (B) Calcium uptake with $^{45}CaCl_2$ (approx. 0.5 μM free Ca^{2+}) and preincubation of sarcoplasmic reticulum vesicles were carried out with 5 nM (▼), 20 nM (▲) calmodulin, 2.5 μg/ml supernatant of boiled sarcoplasmic reticulum (■) or without addition (○) as described in Materials and Methods.

The endogenous calmodulin content of routinely prepared dog-heart sarcoplasmic reticulum fractions [14] used in the present study is about 16.6 pmol/mg (0.282 μg/mg) protein of the sarcoplasmic reticulum fraction. Even if this calmodulin dissociated fully, which is most unlikely, then 1 mg sarcoplasmic reticulum/ml would give a calmodulin concentration of about 16 nM, or a concentration of less than 1 nM in the calcium uptake and ATPase experiments with 0.05 mg sarcoplasmic reticulum protein per ml, which would not be sufficient to activate calcium transport.

The stimulatory effect of calmodulin on the rate of calcium uptake by sarcoplasmic reticulum vesicles declines in response to an elevation in the free calcium concentration in the assay medium. The rate of calcium uptake measured at about 10 μM free Ca^{2+} with sarcoplasmic reticulum vesicles preincubated with 100 nM calmodulin in the pres-

ence of 0.1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM ATP was increased by only 13% as compared with the control in the absence of calmodulin (Table I), indicating that calmodulin increases the apparent calcium affinity to the high-affinity calcium binding sites of the transport ATPase. A similar small effect was observed in presence of 100 mM KCl, whilst the rates of calcium uptake are higher in the presence of 100 mM KCl (Table I).

Fig. 4 shows the effect of calmodulin on the rate of calcium uptake measured with the aid of Arsenazo III [18], which permits a continuous trace of the reaction over the selected time period, but does not allow the selection of low concentrations of free calcium as in the case of calcium plus EGTA in the isotope and filtration method. These experiments, however, clearly demonstrate that the increase in the rate of calcium uptake by calmodulin is due to an increased net uptake and not to a facilitation of calcium-calcium exchange.

Calcium-dependent and calcium-independent ATP hydrolysis by sarcoplasmic reticulum with or without calmodulin was measured under identical conditions as used for the calcium uptake experiments in Fig. 1. Calmodulin increases the rate of calcium-dependent ATP splitting, whilst the rate of calcium-independent ATP splitting remains unchanged (Table I).

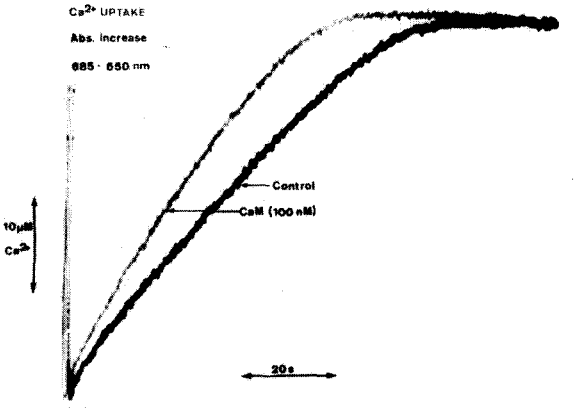

Fig. 4. Stimulation of calcium uptake by calmodulin measured spectrophotometrically with Arsenazo III. The procedure is detailed in Materials and Methods.

Calcium-, calmodulin-dependent phosphorylation of sarcoplasmic reticulum

Fig. 5 shows the time-course of phosphorylation of sarcoplasmic reticulum fractions performed at 25°C, pH 7.0, and a high concentration of free calcium (approx. 10 μM) following preincubation of sarcoplasmic reticulum with 2, 10 and 100 nM calmodulin in the presence of 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$. 100 nM calmodulin causes an addi-

TABLE I

STIMULATION OF THE RATE OF CALCIUM UPTAKE AND THE RATE OF ATP HYDROLYSIS OF SARCOPLASMIC RETICULUM BY 100 nM CALMODULIN

The assay conditions and the preincubation conditions are given in Materials and Methods. The values are quoted as $\mu mol \cdot mg^{-1} \cdot min^{-1}$ and are means ± S.E. for the number of different sarcoplasmic reticulum preparations given in parenthesis. Quoted Ca^{2+} concentrations are approximate.

Conditions		Control	Calmodulin (100 nM)
Ca^{2+} uptake			
0.5 μM Ca^{2+}		0.091 ± 0.009(10)	0.162 ± 0.017(10)
10 μM Ca^{2+}		0.230 ± 0.025 (3)	0.260 ± 0.028 (3)
10 μM Ca^{2+} + 100 mM KCl		0.275 ± 0.023 (3)	0.311 ± 0.026 (3)
ATPase			
0.5 μM Ca^{2+}	total	0.126 ± 0.019 (4)	0.188 ± 0.039 (4)
< 0.01 μM Ca^{2+}	Mg^{2+}-dependent	0.043 ± 0.011 (4)	0.047 ± 0.011 (4)
0.5 μM Ca^{2+}	Ca^{2+},Mg^{2+}-dependent	0.083 ± 0.010 (4)	0.141 ± 0.028 (4)

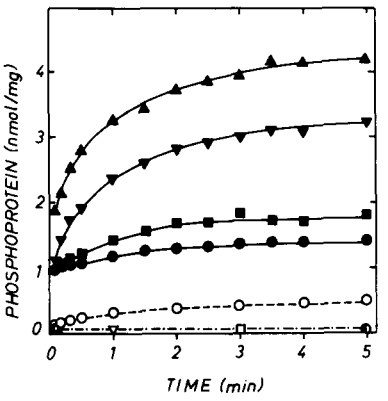

Fig. 5. Time-course of calmodulin-dependent phosphorylation of sarcoplasmic reticulum fractions. Preincubation: 5 min at 25°C, in 20 mM histidine-HCl-Tris (pH 7.0), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2.5 mg sarcoplasmic reticulum protein/ml, and 0 (●), 2 (■), 10 (▼), or 100 nM (▲) calmodulin. Phosphorylation: 25°C; 60 mM histidine-HCl-Tris (pH 7.0), 2 mM phospho*enol*pyruvate, 40 μg/ml pyruvate kinase, 5 mM NaN_3, 10 mM $MgCl_2$, 2 or 4 mM [γ-^{32}P]ATP, 0.5 mM $CaCl_2$, 0.5 mM EGTA, 0 (●), 2 (■), 10 (▼) or 100 nM (▲) calmodulin, 0.17 mg preincubated sarcoplasmic reticulum protein/ml. Zero $CaCl_2$ and zero calmodulin (○), as above but without added $CaCl_2$ and 5 mM EGTA. Zero Ca^{2+}, zero Mg^{2+}: as above, with without added $CaCl_2$ and $MgCl_2$ and 1 mM EGTA plus 1 mM EDTA; control (◐), 2 (□), 10 (▽) or 100 nM (△) calmodulin. Values are means of three experiments with different sarcoplasmic reticulum preparations.

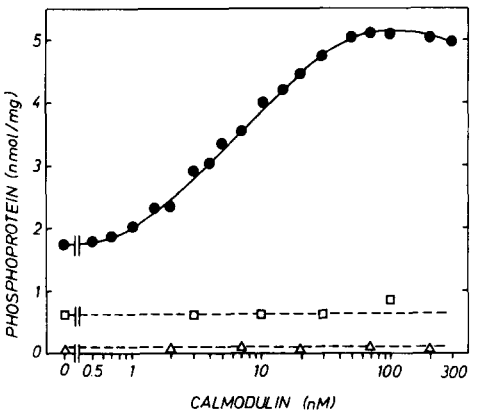

Fig. 6. Dependence of calmodulin-dependent phosphorylation of sarcoplasmic reticulum fractions on calmodulin concentrations. Preincubation of sarcoplasmic reticulum vesicles as in Fig. 5 with 0.5–100 nM calmodulin. Phosphorylation as in Fig. 5 with 4 mM [γ-^{32}P]ATP and calmodulin concentratoins as indicated (●). Zero Ca (□). Zero Ca^{2+}, Zero Mg^{2+} (△). The reaction was started with preincubated sarcoplasmic reticulum and stopped 3 min later. Values are means of two experiments with different sarcoplasmic reticulum preparations.

tional phosphate incorporation of about 3 nmol/mg sarcoplasmic reticulum protein in excess of the phosphorylation of the calcium transport ATPase of sarcoplasmic reticulum. The rate of this calmodulin-dependent phosphorylation, with a half-time of about 20 s at 100 nM calmodulin, is much lower than that of the phosphorylation of the calcium ATPase. Magnesium-dependent phosphate incorporation into sarcoplasmic reticulum fractions amounting to about 0.5 nmol/mg protein was observed in the controls over a very slow time-course; this type of phosphorylation was not investigated further.

The calmodulin dependence of phosphorylation of sarcoplasmic reticulum fractions is shown in Fig. 6. Half-maximal activation of calcium-, calmodulin-dependent phosphorylation is obtained at calmodulin concentrations of about 8 nM.

Calmodulin-dependent phosphorylation of sarcoplasmic reticulum vesicles was also performed in the presence of a low free calcium concentration, of about 0.5 μM, following preincubation of sarcoplasmic reticulum vesicles with calmodulin and magnesium plus or minus calcium, i.e., conditions identical to those used in the calcium uptake experiments in Fig. 1. Preincubation of sarcoplasmic reticulum vesicles with 100 nM calmodulin, 1 mM magnesium and 0.1 mM calcium results in calmodulin-dependent phosphorylation within 1 min to a small, but significant extent, whilst preincubation with 0.1 mM EGTA instead of calcium prevents calmodulin-dependent phosphorylation (Table II). Calcium uptake by sarcoplasmic reticulum vesicles at 1 and 2.5 min, performed under the former preincubation conditions, is shown for comparison (Table II).

Calmodulin-dependent phosphorylation entails the formation of a phosphoester bond [9,11], in contrast to the acylphosphate formed in phosphorylation of the calcium transport ATPase during calcium transport [14]. This is inferred from the hydroxylamine insensitivity of the acid-denaturated calmodulin-dependent phosphoprotein, whilst the acid-denaturated phosphoenzyme is completely decomposed by hydroxylamine (Fig.

TABLE II

CALMODULIN-DEPENDENT PHOSPHORYLATION OF SARCOPLASMIC RETICULUM AT LOW FREE CALCIUM

Preincubation of sarcoplasmic reticulum (SR) vesicles (2.5 mg/ml) was carried out for 2 min at 25°C, pH 7.0, in a medium containing 40 mM histidine-HCl-Tris, and either 0.1 mM $CaCl_2$, 1 mM $MgCl_2$ (mode A) or 0.1 mM EGTA, 1 mM $MgCl_2$ (mode B) in the absence or presence of 100 nM calmodulin. Phosphorylation: 1 min at 25°C; 40 mM histidine-HCl-Tris (pH 7.0), 5 mM NaN_3, 3.5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 0.2 mM $CaCl_2$, 0.3 mM EGTA (approx. 0.5 μM free Ca^{2+}), 0.16 mg preincubated sarcoplasmic reticulum protein/ml with or without 100 nM calmodulin. Calcium uptake by sarcoplasmic reticulum vesicles preincubated under conditions A was performed under identical conditions as phosphorylation except with $^{45}CaCl_2$ and in the presence of 5 mM oxalate. Values are means ± S.E. for the number of different sarcoplasmic reticulum preparations given in parenthesis.

	Phosphoprotein (nmol/mg SR)		Calcium uptake (μmol/mg SR)	
	A	B	A	
	(1 min) (n = 6)	(1 min) (n = 6)	1 min (n = 3)	2.5 min (n = 3)
Control				
0.5 μM Ca^{2+} (approx.)	0.759 ± 0.069	0.586 ± 0.070	0.079 ± 0.003	0.214 ± 0.006
zero Ca, zero Mg	0.062 ± 0.012	0.078 ± 0.016		
Calmodulin (100 nM)				
0.5 μM Ca^{2+} (approx.)	1.045 ± 0.048	0.602 ± 0.052	0.104 ± 0.002	0.290 ± 0.001
zero Ca, zero Mg	0.073 ± 0.018	0.074 ± 0.020		

7). The two types of phosphorylation are further distinguished by the decomposition of the respective phosphoproteins following removal of calcium from the medium by either EGTA or EDTA.

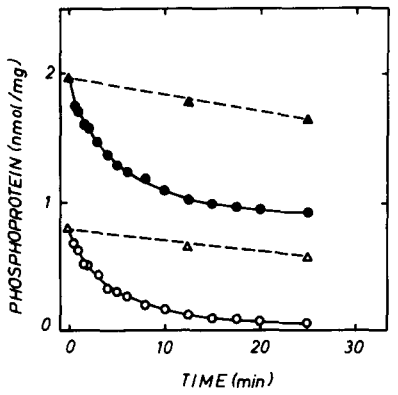

Fig. 7. Hydroxylamine sensitivity of calcium-dependent phosphorylation of sarcoplasmic reticulum fractions in the presence or absence of calmodulin. Phosphorylation was performed for 10 s at 25°C in a medium containing 60 mM histidine-HCl-Tris (pH 7.0), 2 mM phospho*enol*pyruvate, 40 μg/ml pyruvate kinase, 0.5 mM EGTA, 0.5 mM $CaCl_2$ (approx. 10 μM free Ca^{2+}), 0.17 mg sarcoplasmic reticulum protein/ml with 0.3 μM calmodulin (▲, ●) or without calmodulin (△, ○). Hydroxylamine treatment of acid-denaturated phosphoproteins is given in Materials and Methods. (○, ●), 0.4 M hydroxylamine plus 0.2 M sodium acetate buffer (pH 5.2); (△, ▲), 0.2 M sodium acetate buffer (pH 5.2).

TABLE III

FORMATION AND DECOMPOSITION OF CALCIUM-DEPENDENT PHOSPHOPROTEINS OF SARCOPLASMIC RETICULUM FRACTIONS IN THE PRESENCE OR ABSENCE OF CALMODULIN

Phosphorylation was carried out at 25°C in a medium containing 60 mM histidine-HCl-Tris (pH 7.0), 2 mM phospho*enol*-pyruvate, 40 μg/ml pyruvate kinase, 5 mM NaN_3, 5 mM $MgCl_2$, 1 or 2 mM [γ-^{32}P]ATP, 0.5 mM $CaCl_2$, 0.5 mM EGTA (approx. 10 μM free Ca^{2+}), 0.17 mg sarcoplasmic reticulum/ml with or without 300 nM calmodulin. Zero Ca: without addition of calcium, but with 1 mM EGTA. Zero Ca, zero Mg: without addition of $CaCl_2$ and $MgCl_2$, but with 1 mM EGTA plus 1 mM EDTA. 10 s after initiation of phosphorylation by addition of sarcoplasmic reticulum protein 5 mM EGTA or 5 mM EDTA was added and the reaction was stopped 5 s later. Values are means ± S.E. from three different sarcoplasmic reticulum (SR) preparations.

	Phosphoprotein (nmol/mg SR)	
	Control	Calmodulin (300 nM)
Formation (10 s)		
0.5 mM Ca, 0.5 mM EGTA	0.822 ± 0.011	1.886 ± 0.063
zero Ca, 5 mM Mg (1 mM EGTA)	0.092 ± 0.010	0.111 ± 0.021
zero Ca, zero Mg (1 mM EGTA, 1 mM EDTA)	0.047 ± 0.008	0.050 ± 0.005
Decomposition (5 s)		
+ 5 mM EGTA	0.192 ± 0.010	1.298 ± 0.053
+ 5 mM EDTA	0.214 ± 0.027	1.303 ± 0.066

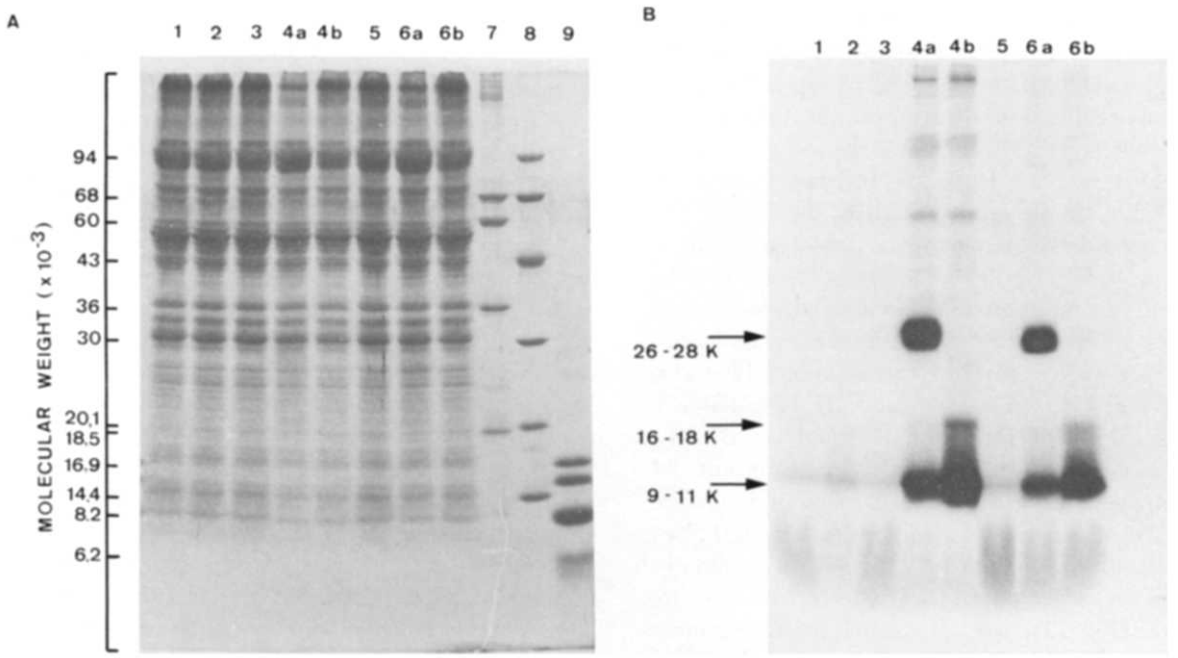

Fig. 8. Electrophoretogram and autoradiogram of phosphorylated sarcoplasmic reticulum fractions in the presence of calmodulin or catalytic subunit of cAMP-dependent protein kinase. A: electrophoretogram; B: autoradiogram. Control (1, 2); 100 nM calmodulin (3, 4, 5); 12 μg/ml catalytic subunit of cAMP-dependent proteinkinase (6). Phosphorylation was carried out for 1 min at 23°C. pH 7.0, in 40 mM histidine-HCl-Tris, 5 mM NaN_3, 3.5 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP, 0.8 mg sarcoplasmic reticulum protein/ml, 0.5 mM EGTA without added calcium (1, 3, 6), or plus 0.5 mM $CaCl_2$ (2, 4, 5) or plus 0.5 mM $CaCl_2$ and 0.5 mM trifluperazine (5). Solubilization prior electrophoresis: 37°C, 20 min (4a, 6a); boiled for 2 min (4b, 6b). The procedure for hydroxylamine treatment is given in Materials and Methods. Standard proteins and their molecular weights (7–9): (7) Ferritin (220000; 18500), albumin (68000), catalase (60000), lactate dehydrogenase (36000). (8) Phosphorylase *b* (94000), albumin (68000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100), α-lactalbumin (14400). (9) Myoglobin (16950), myoglobin I and II (14400), myoglobin I (8159), myoglobin II (6214).

Addition of EGTA or EDTA results in an immediate decomposition of about 0.6 nmol phosphoprotein/mg sarcoplasmic reticulum in the presence or absence of calmodulin (Table III), which is apparently due to the fast decomposition of the major portion of the phosphoprotein formed by the ATPase.

The autoradiogram of an SDS-polyacrylamide gel electrophoretogram of phosphorylated and hydroxylamine-treated sarcoplasmic reticulum fractions shows that several proteins are substrates for calmodulin-dependent phosphorylation (Fig. 8); furthermore, this type of phosphorylation is strongly calcium-dependent and inhibited by trifluperazine (Fig. 8). The most prominently labelled proteins in calmodulin-dependent phosphorylation and also in phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase are a 26–28 kDa and a 9–11 kDa protein when solubilization of the protein is carried out at 37°C prior to electrophoresis. However, boiling for 2 min results in both cases in the disappearance of the 26–28 kDa protein and a marked increase in the labelled 9–11 kDa protein, with the appearance of a 16–18 kDa protein. These three proteins are most probably identical with phospholamban [1–6,9,11,12] and subunits [9,12]. Triton is not required in addition to SDS to achieve subunit dissociation as suggested in Ref. 9.

Discussion

The present study demonstrates a stimulatory effect of low concentrations of calmodulin (5–100

nM) on the rate of calcium uptake and the rate of calcium-dependent ATP hydrolysis by sarcoplasmic reticulum vesicles in association with calcium-, calmodulin-dependent phosphorylation of sarcoplasmic reticulum proteins.

The calmodulin-dependent increase in the rate of calcium uptake and ATP hydrolysis with 100 nM calmodulin assayed at a low free calcium concentration (approx. 0.5 μM) is considered to be due to calmodulin-dependent phosphorylation during preincubation of sarcoplasmic reticulum vesicles in the presence of magnesium, ATP and a higher free calcium concentration (Fig. 1, Table I, Fig. 5). This interpretation is based on the observation that preincubation of sarcoplasmic reticulum vesicles with 100 nM calmodulin in the presence or absence of magnesium and ATP, but without added calcium or with EGTA to remove contaminating calcium, had no effect on calcium uptake (Fig. 1) nor on calmodulin-dependent phosphorylation (Table II) when assayed at low free calcium of about 0.5 μM. The finding that calmodulin has no effect under these conditions (Fig. 1, Table II) may indicate that the formation of the active calmodulin-calcium species necessary for the activation of the membrane-bound, calcium-, calmodulin-dependent protein kinase requires a free calcium concentration greater than 0.5 μM at a total calmodulin concentration of 100 nM. This assumption is supported by the fact that calcium-, calmodulin-dependent phosphorylation is observed at low concentrations of calmodulin without any preincubation of sarcoplasmic reticulum vesicles, if the assay is carried out at a higher free calcium concentration of about 10 μM (Table III, Fig. 8). The above assumption is further supported by recent observations which show that the calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum depends on both the total calmodulin concentration and the free calcium concentration, i.e., the calmodulin dependence of phosphorylation is shifted to lower total calmodulin concentrations at higher free calcium concentrations and the calcium-dependence of calmodulin-dependent phosphorylation is shifted to lower free calcium concentrations at higher total calmodulin concentrations [26]. Additional effects of calcium on calmodulin-dependent phosphorylation are not ruled out, since preincubation of sarcoplasmic reticulum vesicles with 100 nM calmodulin, 1 mM magnesium and 0.1 mM calcium in the absence of ATP causes a small increase in the rate of calcium uptake (Fig. 1, Table II) and results in significant calmodulin-dependent phosphorylation (Table II) when assayed at low free calcium of about 0.5 μM.

The present results show that the rate of calcium transport is stimulated by calmodulin only in association with calmodulin-dependent phosphorylation, but not in its absence, suggesting a causal relationship between these processes.

Activation of calcium transport and calcium-activated ATP hydrolysis by calmodulin at low free calcium concentrations in association with calmodulin-dependent phosphorylation has been reported quite recently [12], but only at calmodulin concentrations which were 10–50-times greater than used in the present study. Those data refer to experiments in which the sarcoplasmic reticulum vesicles were not preincubated with calcium at a high calcium concentration and calmodulin, nor prephosphorylated [12].

The most prominently labelled proteins in calmodulin-dependent phosphorylation, as well as in phosphorylation by the catalytic subunit of cAMP-dependent protein kinase are phospholamban and its subunits (Fig. 8; Refs. 9, 12). Based on the correlation between stimulation of calcium transport and phosphorylation of phospholamban by the cAMP-dependent protein kinase [2], it is most likely that calmodulin-dependent phosphorylation of this protein mediates the calmodulin-dependent stimulation of calcium transport by sarcoplasmic reticulum transport ATPase as proposed by Le Peuch et al. [9]. At least ten proteins present in the crude sarcoplasmic reticulum fraction are phosphorylated in addition to phospholamban and its subunits (Fig. 8). Their functional role is unknown, and some of these proteins may originate from contaminating sarcolemma [6], but the 59 and 35 kDa proteins which undergo calmodulin-dependent phosphorylation may, respectively, be identical with the 60 [27] or 57 [28] and 35 kDa [28] proteins phosphorylated in sarcoplasmic reticulum derived from rabbit skeletal muscle. It has been suggested that the phosphorylation of the 60 or 57 kDa protein may play a role in the calcium release process of

sarcoplasmic reticulum membranes [27,28].

The simple dependence of calmodulin-dependent phosphorylation of sarcoplasmic reticulum on the concentration of exogenous calmodulin at a constant free calcium concentration (Fig. 6) supports the assumption that the membrane-bound calmodulin-dependent protein kinase in our routinely prepared sarcoplasmic reticulum fraction is in a calmodulin-deficient state. Furthermore, hydroxylamine-insensitive phosphorylation of sarcoplasmic reticulum membranes is negligible in the absence of exogenous calmodulin (Fig. 7), which also indicates that the endogenous calmodulin present in the sarcoplasmic reticulum fraction (Fig. 3) is mainly confined to structures other than the calmodulin-dependent protein kinase.

The mechanism responsible for the stimulation of the rate of calcium transport by the sarcoplasmic reticulum ATPase with calmodulin involves an increase in the apparent affinity of calcium to the high-affinity calcium binding sites of the ATPase (Fig. 1, Table I, Ref. 29). The increase in the rate of calcium-activated ATP splitting by calmodulin parallels the increase in the rate of calcium transport, indicating that calmodulin does not affect the calcium transport ratio (Table I, Ref. 12).

Acknowledgements

The authors would like to thank Dr. L. Adler-Kastner for help in the preparation of the manuscript and Mrs. E. Hafrank for typing the manuscript. This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung (Grant 4162 to J.S.), Vienna, Austria.

References

1 Tada, M., Kirchberger, M.A. and Katz, A.M. (1975) J. Biol. Chem. 250, 2640–2647
2 Kirchberger, M.A., Tada, M. and Katz, A.M. (1974) J. Biol. Chem. 249, 6166–6173
3 LaRaia, P.J. and Morkin, E. (1974) Circ. Res. 35, 298–306
4 Wray, H.L. and Gray, R.R. (1977) Biochim. Biophys. Acta 461, 441–459
5 Will, H., Levchenko, T.S., Levitsky, D.O., Smirnov, V.N. and Wollenberger, A. (1978) Biochim. Biophys. Acta 543, 175–193
6 Jones, L.R., Besch, H.R., Jr., Fleming, J.W., Connaughey, M.M. and Watanabe, A.M. (1979) J. Biol. Chem. 254, 530–539
7 Kirchberger, M.A., Tada, M., Repke, D.I. and Katz, A.M. (1972) J. Mol. Cell. Cardiol. 4, 673–680
8 Katz, S. and Remtulla, M.A. (1978) Biochem. Biophys. Res. Commun. 83, 1373–1379
9 Le Peuch, C.J., Haiech, J. and Demaille, J.G. (1979) Biochemistry 18, 5150–5157
10 Lopaschuk, G., Richter, B. and Katz, S. (1980) Biochemistry 19, 5603–5607
11 Kranias, E.G., Bilezikjian, L.M., Potter, J.D., Piascik, M.T. and Schwartz, A. (1980) Ann. N.Y. Acad. Sci. 356, 279–290
12 Kirchberger, M.A. and Antonetz, T. (1982) J. Biol. Chem. 257, 5685–5691
13 Glynn, J.M. and Chappell, J.B. (1964) Biochem. J. 90, 147–149
14 Suko, J. and Hasselbach, W. (1976) Eur. J. Biochem. 64, 123–130
15 Daughaday, W.H., Lowry, O.H., Rosebrough, N.J. and Fields, W.S. (1952) J. Lab. Clin. Med. 39, 663–665
16 Suko, J. (1973) J. Physiol. (Lond.) 238, 563–582
17 Plank, B., Hellmann, G., Punzengruber, C. and Suko, J. (1979) Biochim. Biophys. Acta 550, 259–268
18 Scarpa, A. (1979) Methods Enzymol. 56, 301–338
19 Avron, M. (1960) Biochim. Biophys. Acta 40, 257–272
20 Punzengruber, C., Prager, R., Kolassa, N., Winkler, F. and Suko, J. (1978) Eur. J. Biochem. 92, 349–359
21 Watterson, D.M., Harrelson, W.G., Keller, P.M., Sharief, F. and Vanaman, T.C. (1976) J. Biol. Chem. 251, 4501–4513
22 Cheung, W.Y., Bradham, L.S., Lynch, T.J., Lin, Y.M. and Tallant, E.A. (1975) Biochem. Biophys. Res. Commun. 66, 1055–1066
23 Laemmli, U.K. (1970) Nature 227, 680–685
24 Schwarzenbach, G. and Flaschka, H. (1965) in Die Chemische Analyse (Koch, W., ed.), Vol. 45, p. 9, Ferdinand Enke Verlag, Stuttgart
25 Dedman, J.R., Potter, J.D. and Means, A.R. (1977) J. Biol. Chem. 252, 2437–2440
26 Plank, B., Pifl, C., Hellmann, G., Wyskovsky, W., Hoffmann, R. and Suko, J. (1983) Eur. J. Biochem., in the press
27 Campbell, K.P. and MacLennan, D.H. (1982) J. Biol. Chem. 257, 1238–1246
28 Chiesi, M. and Carafoli, E. (1982) J. Biol. Chem. 257, 984–991
29 Katz, S. (1980) Ann. N.Y. Acad. Sci. 356, 267–278