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CALMODULIN · $(Ca^{2+})_4$ IS THE ACTIVE CALMODULIN-CALCIUM SPECIES ACTIVATING THE CALCIUM-, CALMODULIN-DEPENDENT PROTEIN KINASE OF CARDIAC SARCOPLASMIC RETICULUM IN THE REGULATION OF THE CALCIUM PUMP

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Calcium-, calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum increases the rate of calcium transport. The complex dependence of calmodulin-dependent phosphoester formation on free calcium and total calmodulin concentrations can be satisfactorily explained by assuming that CaM · $(Ca^{2+})_4$ is the sole calmodulin-calcium species which activates the calcium-, calmodulin-dependent, membrane-bound protein kinase. The apparent dissociation constant of the E · CaM · $(Ca^{2+})_4$ complex determined from the calcium dependence of calmodulin-dependent phosphoester formation over a 100-fold range of total calmodulin concentrations (0.01–1 μM) was 0.9 nM; the respective apparent dissociation constant at 0.8 mM free calcium, 1 mM free magnesium with low calmodulin concentrations (0.1–50 nM) was 2.60 nM. These results are in good agreement with the apparent dissociation constant of 2.54 nM of high affinity calmodulin binding determined by ^{125}I-labelled calmodulin binding to sarcoplasmic reticulum fractions at 1 mM free calcium, 1 mM free magnesium and total calmodulin concentration ranging from 0.1 to 150 nM, i.e. conditions where approximately 98% of the total calmodulin is present as CaM · $(Ca^{2+})_4$. The apparent dissociation constant of the calcium-free calmodulin-enzyme complex (E · CaM) is at least 100-fold greater than the apparent dissociation constant of the E · CaM · $(Ca^{2+})_4$ complex, as judged from non-saturation ^{125}I-labelled calmodulin binding at total calmodulin concentrations of up to 150 nM, in the absence of calcium.

Introduction

The calcium binding protein calmodulin [1,2] modifies the calcium transport function of sarcoplasmic reticulum [3,4]. Calmodulin stimulates the rate of calcium transport by cardiac sarcoplasmic reticulum [5–11], which has been suggested to be due to phosphorylation of phospholamban and/or subunits by a calcium-, calmodulin-dependent protein kinase [6]. Indirect evidence for a causal relationship between stimulation of the rate of calcium uptake by cardiac sarcoplasmic reticulum and calmodulin-dependent phosphorylation was obtained recently from the close correlation between the calmodulin-dependent increase in the rate of calcium uptake and the amount of calmodulin-dependent phosphoester formed [11].

The activation of calcium-, calmodulin-dependent phosphorylation by calcium and calmodulin shows a complex dependence on free calcium and total calmodulin, i.e. the apparent K(Ca) decreases with increasing concentrations of total calmodulin

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid.

and the apparent K(CaM) decreases with increasing concentrations of free calcium [11], as has been observed with other calcium, calmodulin-regulated enzymes [12–16]. This pattern of enzyme activation reflects the formation of the activating calmodulin-calcium species, which is dependent on both free calcium and total calmodulin.

The aim of the present study was to identify the active calmodulin-calcium species responsible for the activation of the calmodulin-dependent protein kinase of cardiac sarcoplasmic reticulum which regulates calcium transport by phosphorylation. The data indicate that under the present experimental conditions $CaM \cdot (Ca^{2+})_4$ is the sole activating calmodulin-calcium species of the sarcoplasmic reticulum calmodulin-dependent protein kinase.

Materials and Methods

Reagents. Carrier-free ortho[^{32}P]phosphoric acid and Na^{125}I were purchased from New England Nuclear (Boston); ATP from Sigma Chemical Co. (St. Louis); phospho*enol*pyruvate, pyruvate kinase from Boehringer GmbH (Mannheim); ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA), bovine brain calmodulin from Fluka AG (Buchs); phenyl-Sepharose from Pharmacia (Upsala); all other chemicals were purchased from E. Merck (Darmstadt).

Preparation of sarcoplasmic reticulum. Cardiac sarcoplasmic reticulum fractions were isolated from mongrel dogs [17] and stored at −40°C in a medium containing 10 mM histidine buffer (pH 7.0) and 0.3 M sucrose.

Preparation of calmodulin. Calmodulin was isolated from bovine brain (obtained from the slaughterhouse) by phenyl-Sepharose affinity chromatography according to Gopalakrishna and Anderson [18]. 300 g brain were homogenized in a Waring blender for 30 s in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 1 mM 2-mercaptoethanol and centrifuged at 15000 × *g*. This extraction was repeated once. Isoelectric precipitation (pH 4.3) and a heating step (100°C, 4 min) were carried out prior to separation of calmodulin on a phenyl-sepharose column (1.6 × 9 cm) equilibrated with 50 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol and 0.1 mM $CaCl_2$. The supernatant obtained after the heating step was adjusted to 5 mM $CaCl_2$ and applied to the column, followed by a wash with 15 bed volumes of the above solution and then washed again with this solution containing 0.5 M NaCl. Calmodulin was eluted with a solution containing 50 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol and 1 mM EGTA, dialysed against deionized water and concentrated by lyophylisation.

Preparation of [γ-^{32}P]ATP and ^{125}I-labelled calmodulin. [γ-^{32}P]ATP was prepared according to Glynn and Chappell [19] as described previously [17]. Iodination of calmodulin was performed for 30 min at 25°C with 1 mg calmodulin and 1 mCi Na^{125}I, 90 nmol KI (spec. act. 20000 cpm/1 pmol calmodulin), 1% D-glucose and iodination reagent beds containing lactoperoxidase and glucose oxidase (New England Nuclear). The reaction was stopped by addition of sodium meta-bisulfite, followed by centrifugation at 600 rpm for 15 min to remove the iodination reagent beds. The supernatant was adjusted to 5 mM $CaCl_2$ and applied to a phenyl-Sepharose column (1 ml bed volume) equilibrated with 50 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol and 0.1 mM $CaCl_2$, followed by a wash with 20 bed volumes of the above solution. ^{125}I-labelled calmodulin was eluted with a medium containing 50 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol and 1 mM EGTA, dialysed against deionized water and concentrated by lyophylisation.

Analyses. Protein was measured by the Folin method [20] standardized against bovine serum albumin.

Calmodulin-dependent phosphorylation of sarcoplasmic reticulum fractions was measured with [γ-^{32}P]ATP at 25°C, pH 7.0, and an ionic strength of 0.1 M in a medium containing 203 mM Mops, 5 mM NaN_3, 2 mM EGTA, 0.96–1.92 mM $CaCl_2$, 1.07 mM [γ-^{32}P]ATP, 2.04 mM $MgCl_2$, 0.1–0.25 mg sarcoplasmic reticulum protein/ml without or with 0.01–1 μM total calmodulin (1 mM free Mg^{2+}, 1 mM MgATP and 0.2–5.0 μM free Ca^{2+}). The concentration of MgATP refers to initial concentration; no ATP regenerating system was used in the present study, since ADP which accumulates through ATP hydrolysis (up to about 0.2 mM) has no effect on calmodulin-dependent phosphoester formation [11]. Different conditions

of phosphorylation in the presence of low calmodulin concentrations (0.1–50 nM) are given in the appropriate legends.

Calcium-independent, magnesium-dependent phosphorylation of sarcoplasmic reticulum fractions [10] was performed similarly, but without addition of calcium and in the presence of 1 mM free magnesium. Unspecific phosphate incorporation was measured in the absence of added calcium and magnesium and in the presence of 1–2 mM EGTA and 1–2 mM EDTA [10,11].

The phosphorylation reaction was stopped by addition of EDTA (25 mM final concn.) for 15 s in order to remove the acylphosphate formed by the ATPase [3,4,17] followed by addition of an ice-cold acid solution containing 0.5 M perchloric acid and 0.1 M phosphoric acid [10,11]. The protein was recovered by centrigation, washed once with 10 ml of the latter solution, then transferred to a glass fibre filter (Gelman Sciences Inc., Type A/E), washed again with 25 ml 0.5 M perchloric acid and 100 mM phosphoric acid and 15 ml of the latter solution containing 200 mM NaCl, finally dissolved in 2 ml ethyleneglycol monoethyl ether plus 6 ml Atomlight and the radioactivity counted in a liquid scintillation counter.

^{125}I-labelled calmodulin binding by sarcoplasmic reticulum fractions was measured at 25°C, pH 7.0 in a medium containing 200 mM Mops, 1 mM $MgCl_2$, 0.1 mg sarcoplasmic reticulum/ml and 1 mM $CaCl_2$ or 1 mM EGTA. Incubations were carried out for 30 min. The protein was transferred to a glass fibre filter (Gelman Sciences Inc., Type A/E) and washed with 50 ml 0.1 M sucrose containing 1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (incubation in the presence of 1 mM $CaCl_2$, 1 mM $MgCl_2$) or with 50 ml 0.1 M sucrose containing 1 mM $MgCl_2$, 1 mM EGTA (incubation in the presence of 1 mM $MgCl_2$, 1 mM EGTA). The radioactivity on the filter was counted in a gamma-counter.

Calculation. In order to obtain the desired free calcium, free magnesium and MgATP concentrations for the phosphorylation experiments the requisite total concentrations of calcium, magnesium and ATP at a fixed concentration of EGTA were calculated taking the calcium and magnesium complexes with EGTA and ATP into consideration as described previously [11]. The following association constants were used (log K_a):

$H^+ + EGTA^{4-} \rightleftharpoons HEGTA^{3-}$, 9.46 [21];
$H^+ + HEGTA^{3-} \rightleftharpoons H_2EGTA^{2-}$, 8.85 [21];
$Ca^{2+} + EGTA^{4-} \rightleftharpoons CaEGTA^{2-}$, 11 [21,22];
$Ca^{2+} + HEGTA^{3-} \rightleftharpoons CaHEGTA^{-}$, 5.33 [22];
$Mg^{2+} + EGTA^{4-} \rightleftharpoons MgEGTA^{2-}$, 5.2 [21];
$Mg^{2+} + HEGTA^{3-} \rightleftharpoons MgHEGTA^{-}$, 3.4 [22];
$H^+ + ATP^{4-} \rightleftharpoons HATP^{3-}$, 6.96 [23];
$H^+ + HATP^{3-} \rightleftharpoons H_2ATP^{2-}$, 4.06 [22,24];
$Ca^{2+} + ATP^{4-} \rightleftharpoons CaATP^{2-}$, 3.95 [22,24,26];
$CA^{2+} + HATP^{3-} \rightleftharpoons CaHATP^{-}$, 2.13 [22,24];
$Mg^{2+} + ATP^{4-} \rightleftharpoons MgATP^{2-}$, 4.54 [22,25–27];
$Mg^{2+} + HATP^{3-} \rightleftharpoons MgHATP^{-}$, 2.78 [22,25].

The calmodulin-calcium species were calculated from the intrinsic calmodulin-Ca and calmodulin-Mg dissociation constants given by Haiech et al. [28] which were 0.067, 0.17, 0.6 and 0.9 μM for the $CaM \cdot Ca^{2+}$, $CaM \cdot (Ca^{2+})_2$, $CaM \cdot (Ca^{2+})_3$ and $CaM \cdot (Ca^{2+})_4$ species, respectively, and 70, 270, 100 and 90 μM for the respective calmodulin-Mg complexes. The apparent calmodulin-Ca dissociation constants in the presence of 1 mM free magnesium are 1.024, 0.79, 6.6 and 10.9 μM for the $CaM \cdot Ca^{2+}$, $CaM \cdot (Ca^{2+})_2$, $CaM \cdot (Ca^{2+})_3$ and $CaM \cdot (Ca^{2+})_4$ species, respectively [28]. The use of the above constants is based on the assumptions that these constants determined at pH 7.55 apply also to pH 7.0 (see Ref. 29) and that the buffer used in the present experiments does not interfere with calcium and/or magnesium binding to calmodulin. KCl was omitted in the present experiments in order to simplify the experimental conditions. For calcium binding constants determined in several laboratories see Klee and Vanaman [2] and Keller et al. [29].

The theoretical curves shown in the figures were calculated by fitting the Hill equation including a correction factor for calcium-independent phosphorylation to the experimental data using an iterative, non-linear least squares approximation as described previously [11,30].

Results

Fig. 1 shows the dependence of calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum fractions on the concentration of free calcium carried out at five different total calmodulin concentrations ranging from 0.01 to 1

μM. The acylphosphate formed by the calcium transport ATPase [3,4,17] was removed in these experiments by EDTA treatment for 15 s before the addition of perchloric acid plus phosphoric acid; EDTA has little effect on the phosphoester formed by the membrane-bound, calcium-, calmodulin-dependent protein kinase. The acylphosphate declines on removal of calcium and/or magnesium by EDTA to an extent approaching the level of phosphoprotein formed in the absence of calcium but in the presence of magnesium (Table I; Refs. 11 and 31).

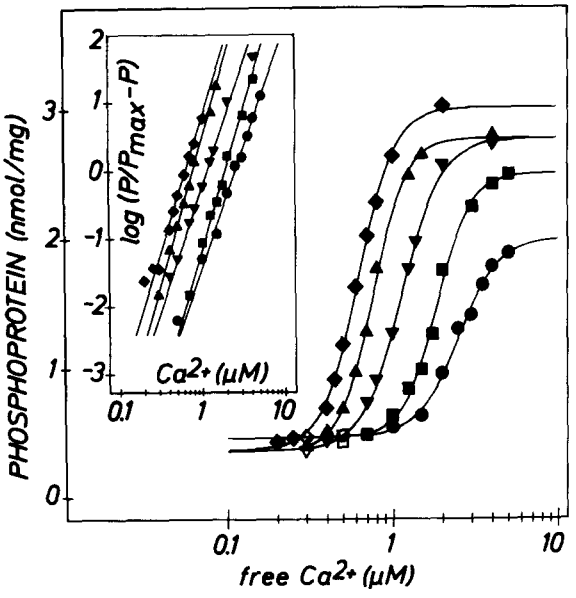

Fig. 1. Calcium dependence of calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum at total calmodulin concentrations from 0.01 to 1 μM. Phosphorylation was performed at 25°C, pH 7.0, 0.1 M ionic strength for 3 min in a medium containing 203 mM Mops, 5 mM NaN_3, 2.04 mM $MgCl_2$, 1.07 mM [^{32}P]ATP, 2 mM EGTA, 0.96–1.92 mM $CaCl_2$ (0.2–5 μM free Ca^{2+}), 0.25 mg sarcoplasmic reticulum/ml without or with 0.01 (●), 0.03 (■), 0.1 (▼), 0.3 (▲) and 1 μM (◆) calmodulin total (1 mM free Mg^{2+}, 1 mM Mg-ATP initially). Zero Ca: as above, but without added $CaCl_2$ and 2 mM EGTA. Zero Ca, zero Mg: as above, but without added $CaCl_2$ and $MgCl_2$, but 2 mM EGTA plus 2 mM EDTA. The reaction was stopped by addition of EDTA (25 mM final concentration) for 15 s before the addition of the acid solution containing 0.5 M perchloric acid and 0.1 M phosphoric acid. Values with zero Ca, zero Ca and zero Mg, and controls plus Ca, Mg are given in Table I. Values are means from two experiments with different sarcoplasmic reticulum preparations. Inset: Hill plot.

The calcium-, calmodulin-dependent phosphoprotein data were analysed by fitting the Hill equation to the data including the calculation of a correction factor for calcium-independent phosphoprotein formation [11,30]. The calculated values for calcium-independent, magnesium-dependent phosphoprotein proved to be very similar to the actually measured values, amounting to about 0.4 nmol/mg sarcoplasmic reticulum protein (3 min incubation; Table I).

Half-maximum activation of calmodulin-dependent phosphorylation at 0.01, 0.03, 0.1, 0.3 and 1 μM total calmodulin occurred at 2.49, 1.86, 1.14, 0.76 and 0.62 μM free calcium i.e. the apparent K(Ca) decreases with increasing total calmodulin concentrations; the Hill coefficients at the above total calmodulin concentrations were 3.59, 4.25, 3.72, 4.39 and 4.05, respectively (Fig. 1). Nearly identical results giving Hill coefficients of about 4, were obtained previously over a smaller range of total calmodulin concentrations under similar conditions, except for the presence of 50 mM KCl [11]. It is to be noted that calmodulin-dependent phosphorylation performed at a fixed concentration of total calmodulin and increasing free calcium concentration shows a plateau over a small range

TABLE I

Mg-DEPENDENT, Ca-, CALMODULIN-INDEPENDENT PHOSPHORYLATION OF CARDIAC SARCOPLASMIC RETICULUM (SR)

Conditions of phosphorylation and EDTA stop for 15 s were performed as in Fig. 1. Control: plus Ca, plus Mg represents mean values obtained at free Ca^{2+} concentrations from 0.2–5 μM (= phosphoprotein after removal of acylphosphate by EDTA treatment [11]). Control: zero Ca plus Mg or zero Ca, zero Mg represent mean values obtained in the absence of calmodulin (CaM). Calmodulin: zero Ca plus Mg or zero Ca, zero Mg represent mean of three or four values obtained with calmodulin concentrations of 0.01, 0.03, 0.1 and 0.3 μM, respectively. Values are means ± S.E. for the number of determinations given in parenthesis.

	Phosphoprotein (nmol/mg SR)
Control (+Ca^{2+}, + Mg^{2+})	0.415 ± 0.011 (21)
Control (0 Ca^{2+}, + Mg^{2+})	0.417 ± 0.019 (8)
CaM (0 Ca^{2+}, + Mg^{2+})	0.394 ± 0.011 (16)
Control (0 Ca^{2+}, 0 Mg^{2+})	0.119 ± 0.015 (7)
CaM (0 Ca^{2+}, 0 Mg^{2+})	0.087 ± 0.005 (15)

of free calcium and declines when the free calcium is further increased [11], which has not been investigated further. Calmodulin-dependent phosphate incorporation 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 and run at alkaline pH (10, 11; see Refs. 6 and 9).

The dependence of calmodulin-dependent phosphorylation of sarcoplasmic reticulum fractions on the concentration of total calmodulin carried out at various fixed free calcium concentrations results in a decrease in the apparent K_m(CaM) with increasing free calcium concentrations measured under similar conditions as in Fig. 1, but in the presence of 50 mM KCl as reported previously [11]; the Hill coefficients for calmodulin-dependence at 1 or 2 μM fixed free calcium were 0.94 and 1.11, respectively [11].

Fig. 2 shows the dependence of calmodulin-dependent phosphoprotein on the calculated CaM · $(Ca^{2+})_4$ species. The concentration of the CaM · $(Ca^{2+})_4$ species formed at free calcium concentrations ranging from 0.2 to 5 μM and total calmodulin concentrations ranging from 0.01 to 1 μM (Fig. 1) have been calculated from the intrinsic calcium and magnesium binding constants given by Haiech et al. [28]; the apparent calmodulin-calcium dissociation constants in the presence of 1 mM free magnesium are 1.02, 0.78, 6.6 and 10.9 μM (see Methods). The apparent dissociation constant (K_a) of the protein kinase CaM · $(Ca^{2+})_4$ complex (E + CaM · $(Ca^{2+})_4 \rightleftharpoons$ E · CaM · $(Ca^{2+})_4$, where E represents the calmodulin-dependent protein kinase) was 0.90 nM as obtained from two experiments with different sarcoplasmic reticulum preparations and a 100-fold variation in the total calmodulin concentration. A value of 0.92 nM was obtained in three experiments with different sarcoplasmic reticulum preparations with a 10-fold variation of the total calmodulin concentration

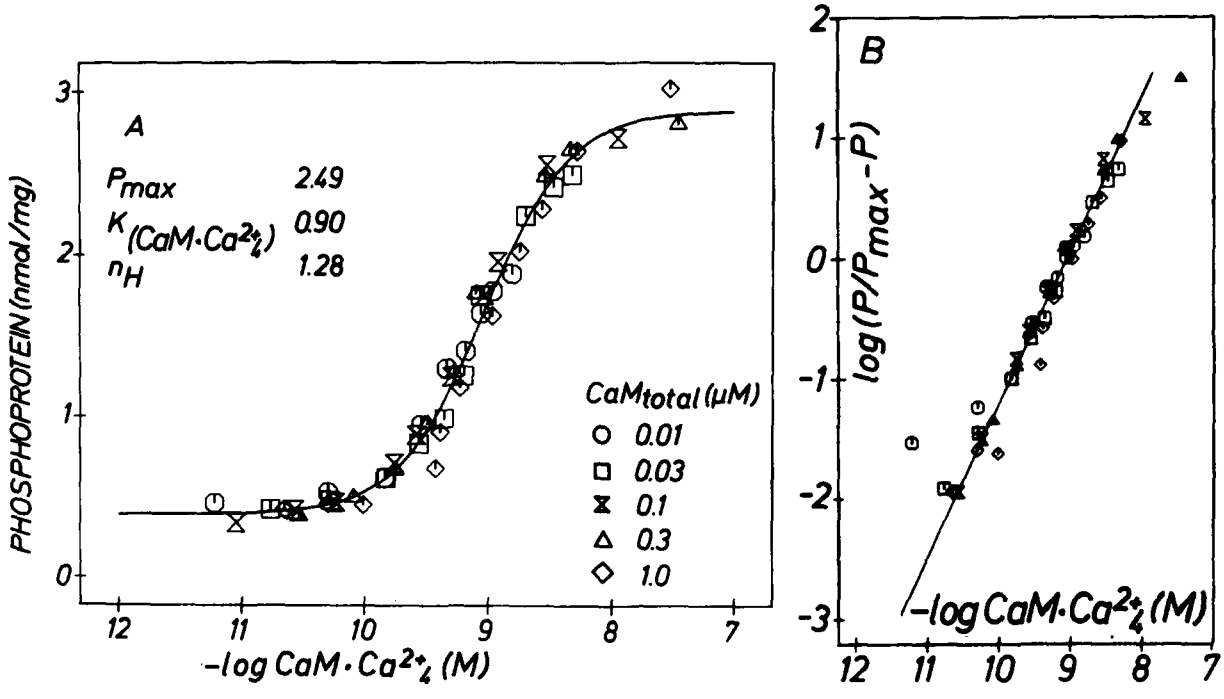

Fig. 2. Dependence of calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum on CaM·$(Ca^{2+})_4$ concentrations with total calmodulin concentrations varying from 0.01 to 1 μM. (A) Phosphoprotein values are those from Fig. 1. CaM·$(Ca^{2+})_4$ concentrations were calculated as described in the Methods. (B) Hill plot. P, phosphoprotein; P_{max}, maximum phosphoprotein; K(CaM·$(Ca^{2+})_4$), concentration of CaM·$(Ca^{2+})_4$ giving half-maximum activation of phosphoprotein formation (nM); n_H, Hill coefficient.

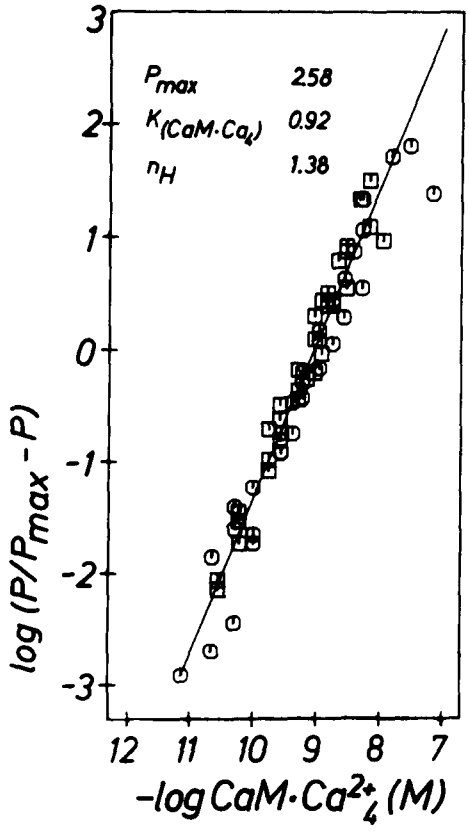

Fig. 3. Dependence of calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum on $CaM \cdot (Ca^{2+})_4$ concentrations of three experiments with total calmodulin concentrations of 0.1 and 1 μM. Conditions of phosphorylation are given in Fig. 1. (○), 0.1 μM calmodulin; (□), 1 μM calmodulin. $CaM \cdot (Ca^{2+})_4$ concentrations were calculated as described in the Methods. Symbols as in Fig. 2.

(0.1 and 1 μM calmodulin; Fig. 3).

Table II shows the distribution of the calmodulin-calcium species calculated for the free calcium concentrations giving half-maximum activation of calmodulin-dependent phosphorylation at the five different total calmodulin concentrations shown in Fig. 1. The concentrations of the $CaM \cdot (Ca^{2+})_4$ species showed little variation (2.6-fold, ranging from 0.45 to 1.18 nM) when the total calmodulin concentration was varied over a 100-fold range. The concentrations of the $CaM \cdot (Ca^{2+})_3$ and $CaM \cdot (Ca^{2+})_2$ species varied 10-fold and 40-fold, respectively.

Similar results for the apparent K_a of the protein kinase-$CaM \cdot (Ca^{2+})_4$ complex were obtained when calmodulin-dependent phosphorylation was measured at a fixed, high free calcium concentration of 0.8 mM, in the presence of 1 and 5 mM free magnesium and total calmodulin concencentrations variing between 0.1 and 50 nM (Fig. 4; Table III). The maximum calmodulin-dependent phosphoprotein obtained with 5 mM free magnesium was greater than with 1 mM free magnesium in incubations carried out for 10 min since the rate of phosphorylation is faster under the former conditions. This is probably due to the fact that the concentration of Ca-ATP is considerably smaller in the presence of 5 mM free magnesium (0.04 mM) than with 1 mM free magnesium (0.21 mM). Ca-ATP is not a substrate for the calmodulin-dependent protein kinase and probably inhibits the binding of Mg-ATP. The apparent K_a value

TABLE II

CONCENTRATION OF THE CALMODULIN-CALCIUM SPECIES AT K(Ca) OF CALMODULIN-DEPENDENT PHOSPHORYLATION OF CARDIAC SARCOPLASMIC RETICULUM AT DIFFERENT TOTAL CALMODULIN CONCENTRATIONS

The free Ca^{2+} concentrations at half-maximum activation of calmodulin-dependent phosphorylation at 0.01, 0.03, 0.1, 0.3 and 1 μM total calmodulin (CaM) are those obtained from Fig. 1. Calculation of calmodulin-Ca^{2+} species is given in the Methods.

CaM_{total} (μM)	$K(Ca^{2+})$ (μM)	$CaM \cdot Ca^{2+}$ (nM)	$CaM \cdot (Ca^{2+})_2$ (nM)	$CaM \cdot (Ca^{2+})_3$ (nM)	$CaM \cdot (Ca^{2+})_4$ (nM)
0.01	2.49	–	0.04	0.16	0.45
0.03	1.86	0.01	0.11	0.35	0.72
0.10	1.14	0.04	0.30	0.56	0.72
0.30	0.76	0.14	0.63	0.80	0.68
1.00	0.62	0.45	1.66	1.72	1.18

TABLE III

CALMODULIN-DEPENDENCE OF CALMODULIN-DEPENDENT PHOSPHORYLATION AT 0.8 mM FREE Ca^{2+}, 1 mM OR 5 mM FREE Mg^{2+} AND LOW TOTAL CALMODULIN CONCENTRATIONS

Conditions of phosphorylation as in Fig. 4. Values are means ± S.E. for the number of determinations given in parenthesis. CaM, calmodulin; SR, sarcoplasmic reticulum.

	Maximum CaM-dep. phosphoprotein (nmol/mg SR)	$K(CaM_{total})$ (nM)
0.8 mM Ca^{2+}, 1 mM Mg^{2+}	1.77 ± 0.11 (8)	2.60 ± 0.38 (8)
0.8 mM Ca^{2+}, 5 mM Mg^{2+}	2.60 ± 0.30 (4)	3.45 ± 0.42 (4)

obtained in the presence of 0.8 mM free calcium, 1 mM free magnesium and low calmodulin concentrations (where about 98% of the total calmodulin being present as $CaM \cdot (Ca^{2+})_4$, calculated according to Ref. 28) was 2.60 nM, which is in the range of the K_a value obtained at low free calcium and high calmodulin concentrations (Fig. 1; Table III).

In order to substantiate the apparent K_a obtained in experiments on calmodulin-dependent phosphorylation, calmodulin binding by cardiac sarcoplasmic reticulum vesicles was measured in the presence of 1 mM EGTA, 1 mM magnesium (free calcium < 0.01 μM) or in the presence of 1 mM calcium, 1 mM magnesium using ^{125}I-labelled calmodulin.

High-affinity calmodulin binding was observed in the presence of calcium, with an apparent K_d of 2.54 nM (range: 1.4–3.7 nM), which must be due to the binding of calmodulin $\cdot (Ca^{2+})_4$ which is the

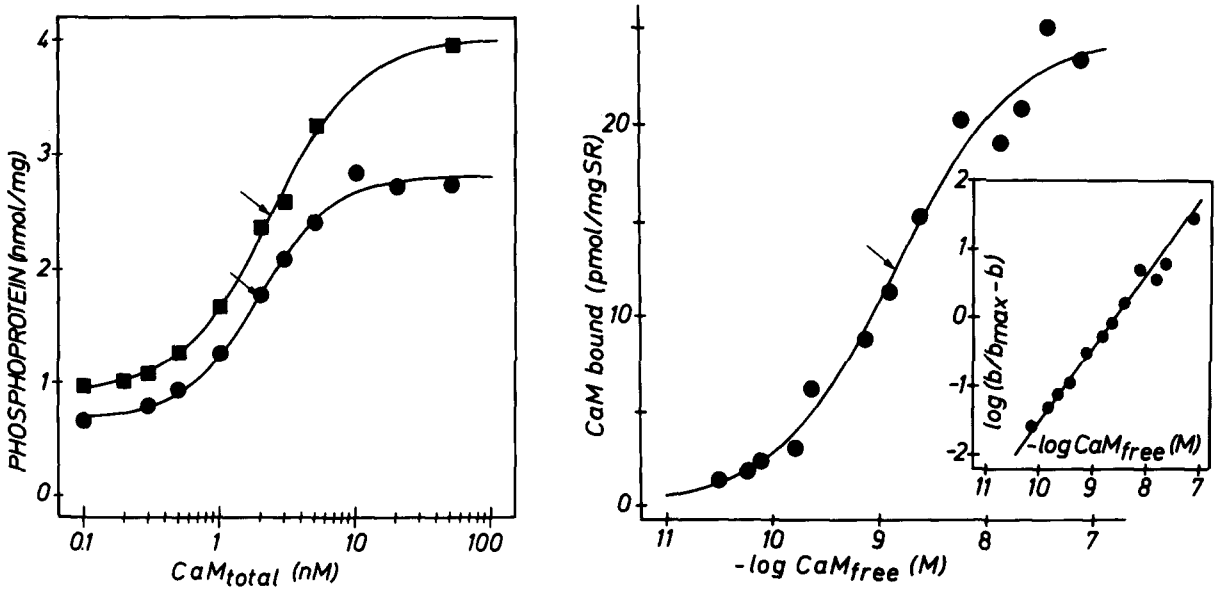

Fig. 4. Calmodulin-dependence of calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum at 0.8 mM Ca^{2+} and low total calmodulin concentrations from 0.1 to 50 nM. Phosphorylation was performed at 25°C (pH 7.0), 0.1 M ionic strength for 10 min in a medium containing 182–208 mM Mops, 5 mM NaN_3, 0.06–0.25 mg sarcoplasmic reticulum/ml, 0.1–50 nM calmodulin total, 1.27 mM [^{32}P]ATP, 2.02 mM $MgCl_2$, 1.01 mM $CaCl_2$ (0.8 mM free Ca, 1 mM free Mg^{2+}, 1 mM Mg-ATP, 0.21 mM Ca-ATP initially) (●), or 1.07 mM [^{32}P]ATP, 6.02 mM $MgCl_2$, 0.84 mM $CaCl_2$ (0.8 mM free Ca^{2+}, 5 mM free Mg^{2+}, 1 mM Mg-ATP and 0.04 mM Ca-ATP initially) (■). Arrows indicate $K(CaM_{total})$.

Fig. 5. ^{125}I-labelled calmodulin binding to cardiac sarcoplasmic reticulum. Calmodulin binding was performed as described in the Methods. Abscissa: $CaM_{free} = CaM_{total}$ minus $CaM_{bound\ total}$. Arrow indicates K(CaM).

TABLE IV

CALCIUM-DEPENDENT ^{125}I-LABELLED CALMODULIN BINDING BY CARDIAC SARCOPLASMIC RETICULUM

Conditions are given in the Methods. $CaM_{bound\ max}$, maximum calmodulin bound; $K(CaM_{free})$, free calmodulin concentration at $CaM_{bound\ max}/2$; n_H, Hill coefficient.

	$CaM_{bound\ max}$ (pmol/mg SR)	$K(CaM_{free})$ (nM)	n_H
Expt. 1	24.54	1.38	0.78
Expt. 2	57.30	2.54	0.87
Expt. 3	49.72	3.69	0.62

most abundant species present under these conditions (Fig. 5; Table IV). Calcium-independent calmodulin binding was small and non-saturable in three of four experiments. The amount of calcium-independent calmodulin binding to sarcoplasmic reticulum vesicles at concentrations giving half-maximum calcium-dependent calmodulin binding was less than 5%.

For the present investigation it is not important if calmodulin binding occurs to more than one target protein present in the sarcoplasmic reticulum fraction having similar high affinities. However, the close similarity between the K_d for high-affinity calmodulin Ca binding and the apparent K_a values determined from phosphorylation experiments suggests that both experimental procedures give a value of the correct order of magnitude for the apparent dissociation constant of the protein kinase-CaM $\cdot (Ca^{2+})_4$ complex.

Discussion

The present study indicates that the complex dependence on free calcium and total calmodulin of calcium-, calmodulin-dependent phosphoester formation by cardiac sarcoplasmic reticulum is due to the activation of the calmodulin-dependent protein kinase by the CaM $\cdot (Ca^{2+})_4$ species. The general reaction scheme of the formation of the calmodulin-calcium species and calmodulin-calcium-enzyme complexes given by Huang et al. [12] for the analysis of cyclic nucleotide phosphodiesterase activation should apply to calmodulin-regulated protein kinases as well and is shown in Fig. 6 to facilitate the present discussion.

The postulation that $CaM \cdot Ca_4^{2+}$ is the only activating calmodulin-calcium species is based on the following findings: The activation of calmodulin-dependent phosphorylation by free calcium at a fixed concentration of total calmodulin depends on the fourth power of free calcium (Fig. 1). Calmodulin-dependent phosphorylation determined at calmodulin concentrations ranging from 0.01 to 1 μM and a low free calcium (0.2–5 μM) is fitted by a single activation curve depending on the concentration of the calculated CaM $\cdot (Ca^{2+})_4$ species (using the apparent dissociation constants K_1–K_4 appropriate to a free magnesium concentration of 1 mM) with an apparent dissociation constant (K_a) of 0.90–0.92 nM (Figs. 2 and 3). A similar K_a of 2.60 nM was obtained from phosphorylation experiments at low total calmodulin concentrations (0.1–50 nM) and a high free calcium concentration of 0.8 mM, i.e. conditions where the concentration of total calmodulin and CaM $\cdot (Ca^{2+})_4$ are almost identical (Fig. 4). It is noted that the determination of the dissociation constant K_a under the former conditions depends greatly on the apparent dissociation constants K_1–K_4 used

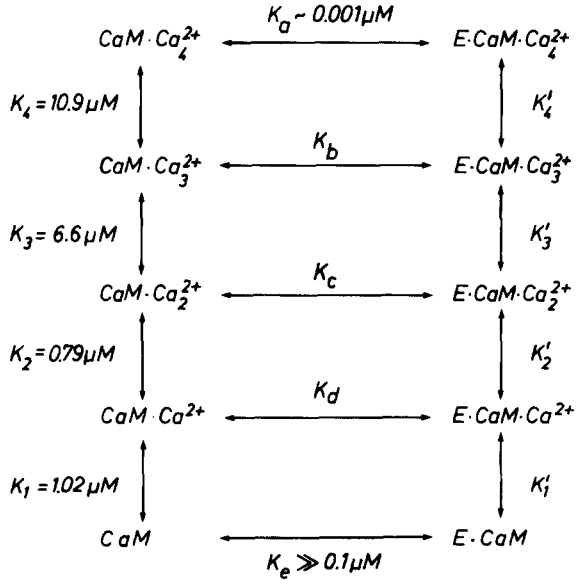

Fig. 6. Reaction scheme on the formation of CaM $\cdot Ca^{2+}$ species and E $\cdot$ CaM $\cdot Ca^{2+}$ species according to Huang et al. [12]. E, Ca^{2+}-, calmodulin-dependent protein kinase; K_1, K_2, K_3, K_4, apparent dissociation constants of the indicated calmodulin-calcium species calculated according [28], accounting for 1 mM free Mg^{2+}. K_a and K_e values, see text.

for the calculation of the calmodulin-calcium species (see Methods; Fig. 6), but not under the latter conditions of a high free calcium concentration of 0.8 mM. The apparent dissociation constant of the $E \cdot CaM \cdot (Ca^{2+})_4$ complex determined from phosphorylation experiments (Figs. 2–4; Table III) is in good agreement with the apparent K_d of 2.54 nM for the high affinity binding of ^{125}I-labelled calmodulin (Fig. 5; Table IV). The binding data indicate that under the selected conditions (1 mM Ca^{2+}, 1 mM Mg^{2+}) the $CaM \cdot (Ca^{2+})_4$ species is bound to the sarcoplasmic reticulum membranes since this species represents more than 98% of the total calmodulin [28]. The binding data, however, do not rule out a binding of not fully calcium-saturated calmodulin-calcium species with high affinity [2,32], which has not been determined in the present investigation.

$CaM \cdot (Ca^{2+})_4$ has been suggested to be the only calmodulin-calcium species activating brain cyclic nucleotide phosphodiesterase [12,33] and myosin light chain kinase [13], as proposed in the present study for the calcium-, calmodulin-dependent protein kinase of cardiac sarcoplasmic reticulum. However the activation of myosin light chain kinase by only two calciums bound to calmodulin has been reported [34]. In the presence of a higher ionic strength Cox and coworkers determined that both $CaM \cdot (Ca^{2+})_3$ and $CaM \cdot (Ca^{2+})_4$ activate cyclic nucleotide phosphodiesterase [14], $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocytes [15] and brain adenylate cyclase [16], whilst $CaM \cdot (Ca^{2+})_3$ was found to be the main activating calmodulin-calcium species.

The present data indicate that the calmodulin-dependent protein kinase has a very high affinity for the activating $CaM \cdot (Ca^{2+})_4$ species with an apparent dissociation constant of about 1 nM, which is somewhat greater than obtained for the cyclic nucleotide phosphodiesterase [12] and similar to the value reported for the myosin light chain kinase [13]. The apparent affinity of calcium-free calmodulin however, is much lower than the apparent affinity for the activating $CaM \cdot (Ca^{2+})_4$ species. From a comparison of calmodulin binding by sarcoplasmic reticulum fractions in the presence of 1 mM free calcium plus 1 mM magnesium with the findings in the absence of free calcium (< 0.01 μM), it appears, assuming Michaelis-type binding, that the dissociation constant of calcium free calmodulin (K_e) is at least two orders of magnitude greater than the apparent K_a. However, the K_e value may well be even greater if, for example, calmodulin binding in the absence of free calcium represents the sum of low-affinity binding to the calmodulin compartment saturated by $CaM \cdot (Ca^{2+})_4$ and low affinity binding to other structures of higher binding capacity. A value of greater than 10 μM has been postulated for brain cyclic nucleotide phosphodiesterase [12] and a value of 25 μM for the $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocytes [35].

If the data of the present study performed in the presence of a physiological magnesium concentration, but in the absence of KCl are relevant for the activation of the calmodulin-dependent protein kinase in vivo it follows that during relaxation of the heart muscle at an approximate calcium concentration of about 0.1 μM, half-maximum activation of the calcium-, calmodulin-dependent protein kinase would require total calmodulin concentrations of about 100 μM, which is about 10-times greater than the calmodulin concentration found in calmodulin reach organs such as brain and testis [36]. This consideration suggests that the calmodulin-dependent protein kinase of cardiac sarcoplasmic reticulum is in a deactivated state during relaxation.

The postulation of a beat to beat activation of the calmodulin-dependent regulatory system of cardiac sarcoplasmic reticulum [9] requires more knowledge of the initial rates of calmodulin-dependent phosphorylation at physiological calmodulin concentrations and dephosphorylation of calmodulin-dependently formed phosphoesters. Recent investigations show the presence of an endogenous phosphatase in our crude sarcoplasmic reticulum fraction which dephosphorylates calmodulin-dependently formed phosphoesters (unpublished results), but the rates of dephosphorylation obtained as yet in vitro are far to slow.

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