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Ca^{2+} Regulation of Sarcoplasmic Reticular Protein Phosphatase Activity†

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ABSTRACT: Isolated rabbit skeletal muscle sarcoplasmic reticulum contains Ca^{2+}-dependent protein kinase and protein phosphatase activities which may regulate the Ca^{2+} transport ATPase [Hörl, W. H., & Heilmeyer, L. M. G., Jr. (1978) *Biochemistry 17*, 766–772; Hörl, W. H., Jennissen, H. P., & Heilmeyer, L. M. G., Jr. (1978) *Biochemistry 17*, 759–766]. Addition of ATP–Mg^{2+} to the concentrated suspension of sarcoplasmic reticulum in the presence of 10 μM Ca^{2+} inhibits the endogenous protein phosphatase activity to 74–90%. This inhibition can be reversed by reducing the free Ca^{2+} concentration with ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N'N'*-tetraacetic acid (EGTA) or by high dilution of the membrane suspension. Kinetically, 10 μM Ca^{2+} increases the K_m' for troponin about 10-fold without any change in V_{max}. Dilution of the membranes in the presence of Ca^{2+} (10 μM) leads to an approximately sevenfold decrease in the K_m' for phosphorylase *a* and an approximately fourfold increase in V_{max}; in the absence of Ca^{2+} (10 nM) no change in K_m' is observed and the V_{max} increases approximately two- to threefold. Membranes without the ATP–Mg^{2+} step during preparation do not show the Ca^{2+}-induced protein phosphatase inhibition; however, after preincubation with the catalytic subunit of the cyclic AMP dependent protein kinase and ATP–Mg^{2+}, the Ca^{2+}-dependent inhibition of the endogenous protein phosphatase reappears. Centrifugation experiments show that the association of the protein phosphatase to the membranes is Ca^{2+} and protein concentration dependent. In the presence of 10 μM Ca^{2+} and at a protein concentration higher than 5 mg/mL, approximately half the amount of phosphatase activity remains in the supernatant in comparison to 10 nM Ca^{2+}.

A Ca^{2+}-dependent protein kinase which is similar but probably not identical with phosphorylase kinase[1] and a protein phosphatase are associated with isolated rabbit skeletal muscle sarcoplasmic reticulum. These two enzymes may regulate the Ca^{2+} transport ATPase activity (Hörl et al., 1978; Hörl &

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[1] Enzymes: phosphorylase kinase (ATP:phosphorylase *b* phosphotransferase, EC 2.7.1.38); phosphorylase *b* or 1,4-γ-glucan:orthophosphate γ-glycosyltransferase (EC 2.4.1.1); phosphorylase phosphatase or phosphorylase phosphohydrolase (EC 3.1.3.17); ATPase or ATP phosphohydrolase (EC 3.6.1.3).

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Heilmeyer, 1978; Varsányi et al., 1978). Phosphorylase kinase (Dickneite et al., 1978; M. W. Kilimann and L. M. G. Heilmeyer, Jr., unpublished experiments) and phosphorylase phosphatase may represent multifunctional enzymes. In addition to phosphorylase *a*, the phosphatase dephosphorylates glycogen synthetase *b*, the phosphorylated β subunit of phosphorylase kinase (Antoniw et al., 1977), the phosphorylated inhibitor subunit of troponin (England et al., 1972), and phosphorylated histone (Nakai & Thomas, 1973; Antoniw et al., 1977). Protein phosphatases with broad substrate specificity probably exist also in other organs (Khandelwal et al., 1976; Killilea et al., 1976) and other species (Li et al., 1978). The protein phosphatase which is associated with the membranes of sarcoplasmic reticulum accepts troponin and phosphorylase *a* as substrates (Hörl & Heilmeyer, 1978; Varsányi et al., 1978).

A material which contains both elements of the sarcoplasmic reticulum and all enzymes involved in the glycogen metabolism and its regulation can be isolated as a protein–glycogen complex (Meyer et al., 1970; Heilmeyer et al., 1970; Haschke et al., 1970, 1972; Caudwell et al., 1978; Detwiler et al., 1977). It represents an organizational level of the enzymes between that of the intact cell and the purified proteins. Enzymes which are integrated in this complex do not behave identically with those in the isolated purified state.

One of the main unsolved problems concerns the Ca^{2+}-dependent inhibition of phosphorylase phosphatase in this particulate fraction (Haschke et al., 1970). During the flash activation of phosphorylase *b* (Heilmeyer et al., 1970; Detwiler et al., 1977; Heilmeyer & Haschke, 1972), which is initiated by ATP–Mg^{2+} and 10 μM Ca^{2+}, the phosphorylase phosphatase activity is transiently inhibited to ~90%. Low molecular weight products like AMP, IMP, etc., which are produced and which are potent inhibitors of the phosphatase reaction if purified phosphorylase *a* is used as substrate (Bot & Dősa, 1971; Bailey & Whelan, 1972; Martensen et al., 1973; Nolan et al., 1964), are not responsible for this phenomenon (Haschke et al., 1970, 1972). Also, covalent modification of the phosphatase seems to be improbable (Haschke et al., 1970).

The K_m' for phosphorylase *a* decreases upon dilution of purified phosphorylase phosphatase (Detwiler et al., 1977). A similar observation was made when the protein–glycogen complex (Haschke et al., 1972) was diluted, which, however, leads to a loss of the Ca^{2+}-dependent phosphatase inhibition during flash activation.

Recently characterized heat-stable polypeptides, one of which exists in a phosphorylated and a nonphosphorylated form, inhibit phosphorylase phosphatase (Huang & Glinsmann, 1975, 1976a,b; Cohen et al., 1977; Tóth et al., 1977). In addition, phosphophosphorylase kinase or dephosphophosphorylase kinase (Bot et al., 1975; Gergely et al., 1976) or the catalytic subunit of the cyclic AMP dependent protein kinase (Gergely & Bot, 1977) may also be an inhibitor of this enzyme. However, all of these inhibitors are probably not responsible for the transient phosphatase inhibition since no effect of 10 μM Ca^{2+} has been observed.

If the same protein phosphatase is involved in the regulation of glycogenolysis and Ca^{2+} transport, this enzyme would have to be present in two compartments: (1) the membranes of the sarcoplasmic reticulum and (2) the protein–glycogen complex. Therefore, the behavior of the sarcoplasmic reticular protein phosphatase was studied. The present publication will show that 10 μM Ca^{2+} inhibits the membrane-associated phosphatase activity. This inhibition is abolished by a decrease of the Ca^{2+} concentration to 10 nM; concomitantly, a reversible dissociation–association of this enzyme with the membranes occurs.

Experimental Procedure

Vesicles of the sarcoplasmic reticulum from rabbit skeletal muscle were prepared according to De Meis & Hasselbach (1971).

Rabbit skeletal muscle phosphorylase kinase was prepared according to Cohen (1973) and as modified by Jennissen & Heilmeyer (1975). The enzyme had a specific activity at pH 8.2 of ~8000 nmol of phosphatase transferred/[min (mg of protein)].

Phosphorylase phosphatase from rabbit skeletal muscle (~60 units/mg) was prepared according to Djovkar (1974). Freshly excised mixed rabbit skeletal muscle was homogenized in 2.5 volumes of 20 mM Tris, 0.1 mM $MnCl_2$, 4 mM EGTA,[2] 1 mM DTE, and 0.01 mM PMSF in a Waring Blendor for 45 s. The homogenate was adjusted with 1 M Tris to pH 7.0 and centrifuged at 15000*g* for 40 min. After a 1:1 dilution of the supernatant (crude extract) with 40% sucrose, 4 mM EGTA, 0.1 mM PMSF, 2 mM DTE, 0.2 mM $MnCl_2$, and 20 mM sodium glycerolphosphate, pH 7.0, the mixture was filtrated over methylamine-substituted Sepharose 4B [15 μmol of methylamine/mL of packed Sepharose; compare Jennissen & Heilmeyer (1975)]. Approximately 90% of the phosphorylase phosphatase activity was retained and eluted with 10 mM sodium glycerol phosphate, 1 mM DTE, 2 mM EGTA, 0.05 mM PMSF, 0.1 mM $MnCl_2$, and 200 mM NaCl, pH 7.0. The eluate was concentrated ca. 10-fold over an Amicon XM 50 filter. The phosphatase was separated from phosphorylase kinase by gel filtration over Sepharose 4B equilibrated in 50 mM Tris-HCl, 1 mM DTE, and 0.1 mM $MnCl_2$, pH 7.5. The enzyme was enriched ca. 70-fold with an average yield of 45%.

Activity assays were carried out according to Haschke et al. (1970).

Troponin was prepared according to Straprans et al. (1972) and as modified by Sperling et al. (1978).

The catalytic subunit of the cyclic AMP dependent protein kinase [470 nmol/(min mg)] was prepared according to Beavo et al. (1974).

[γ-^{32}P]ATP was prepared according to Glynn & Chapell (1964).

[^{32}P]Phosphate was obtained from Amersham Buchler, Braunschweig.

Protein was determined by the method of Lowry (1951) on a Technicon autoanalyzer by using bovine serum albumin as a standard ($A_{280nm}^{1\%} = 4.42 \times 10^4$ M^{-1} cm^{-1}).

Radioactivity was determined as described by Varsányi et al. (1978).

Phosphorylation of Holotroponin on the T Subunit by Rabbit Skeletal Muscle Phosphorylase Kinase. An incubation mixture of 8.3 mL contained 52 mg of holotroponin, 2.86 mg of rabbit skeletal muscle phosphorylase kinase, 5 mM [γ-^{32}P]ATP, 10 mM magnesium acetate, 50 mM Tris-HCl, and 20 mM mercaptoethanol, pH 7.5. During the incubation (1 h, 30 °C) samples of 10 μL were withdrawn, and the protein-bound radioactivity was determined according to Mans & Novelli (1961). In a control reaction the autophosphorylation of an equivalent amount of muscle phosphorylase kinase was determined and taken in correction. To separate the phosphorylated troponin from the phosphorylated kinase

[2] Abbreviations used: EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Cl_3AcOH, trichloroacetic acid; DTE, 1,4-dithioerythritol; PMSF, phenylmethanesulfonyl fluoride.

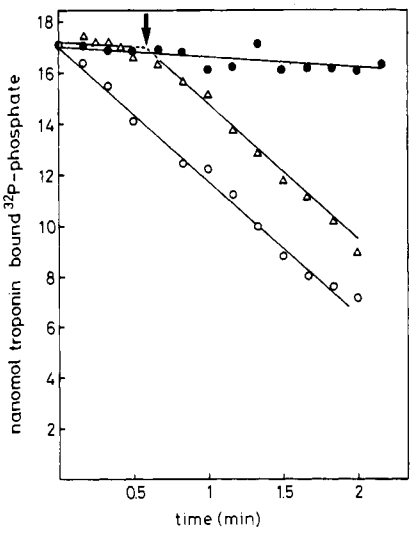

FIGURE 1: Dephosphorylation of phosphorylated troponin by the protein phosphatase associated with the membranes of sarcoplasmic reticulum. Dephosphorylation was carried out in the presence of ~10 μM (●) and ~10 nM (○) Ca^{2+}. 1 mM EGTA (△) was added to the incubation mixture containing 10 μM free Ca^{2+} at the arrow. The loss of protein-bound radioactivity was determined as described by method II.

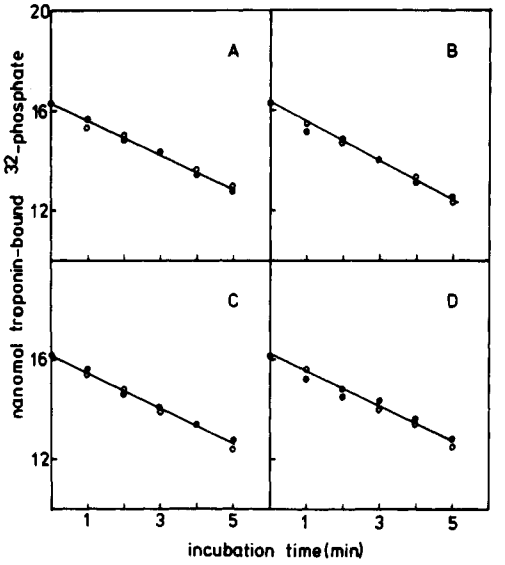

FIGURE 2: Dephosphorylation of phosphorylated holotroponin by partial purified rabbit skeletal muscle protein phosphatase. Dephosphorylations were carried out in the presence of ~10 μM (●) and ~10 nM (○) Ca^{2+}. One milliliter of incubation mixture contained 1.2 mg of labeled troponin and 48.25 μg of protein phosphatase in the presence of 20 mM Tris-HCl and 1 mM DTE, pH 7.5. The loss of protein-bound radioativity was assayed according to Mans & Novelli (1961). (A) Without effectors; (B) in the presence of 1 mM AMP; (C) in the presence of 5 mM ATP + 10 mM Mg^{2+}; (D) in the presence of 5 mM ATP + 10 mM Mg^{2+} and 40 μg of purified rabbit skeletal muscle phosphorylase kinase.

and [γ-^{32}P]ATP, the incubation mixture was filtered through Sepharose 6B. Troponin was eluted in 20 mM Tris-HCl and 1 mM DTE, pH 7.5. The pool from the Sepharose 6B column was concentrated on an Amicon PM 10 filter and stored at −18 °C. The isolated troponin (2.5–2.8 mg/mL) contained 0.12–0.90 μCi/μg (0.11–0.85 mol of [^{32}P]phosphate/90 000 g).

Labeled phosphorylase a (0.12 μCi/mg) was prepared according to Krebs et al. (1958).

Assay of Protein Phosphatase Activity. For the calculation of the sarcoplasmic reticular protein phosphatase activity, initial velocities were assayed at pH 7.5 and 30 °C from aliquots removed usually 6 times during the incubation (not more than 30% dephosphorylation) in two ways.

Method I. One milligram per milliliter labeled phosphorylase *a* was incubated with a specified amount of vesicles of sarcoplasmic reticulum in the presence of 20 mM Tris-HCl and 1 mM DTE, pH 7.5. In the following, the tests were carried out according to Haschke et al. (1970) (method I).

Method II. Holotroponin, phosphorylated on the T subunit, was incubated with vesicles of sarcoplasmic reticulum or partially purified protein phosphatase. One milliliter of incubation mixture contained 0.6–0.8 mg of labeled troponin, ~10 mg of sarcoplasmic reticulum or purified phosphatase, 50 mM KCl, 50 mM Tris-HCl, an equimolar amount of Ca^{2+} and EGTA (1 mM; ~10 μM free Ca^{2+}) or 1 mM EGTA (~10 nM free Ca^{2+}), 5 mM ATP, 10 mM Mg^{2+}, and 40 μg of purified phosphorylase kinase. The reaction was started 5 s after the addition of ATP-Mg^{2+} with phosphorylated troponin. Samples of 100 μL were withdrawn and added to 500 μL of 10% Cl_3AcOH–2 mM ATP–0.5 mM KH_2PO_4. After being allowed to stand at 0 °C for 10 min, the precipitates were filtered through Millipore by using Whatman glass microfiber paper (ϕ 2.5 cm); the papers were further washed as described by Mans & Novelli (1961). Alternatively, samples of 25 μL were withdrawn and applied directly to filter papers (Whatman GF/C ϕ 25 mm) for estimation of protein-bound ^{32}P.

Results

Ca^{2+} Dependent Inhibition of Membrane-Associated Protein Phosphatase. Similar to the conditions of the flash activation test, the protein phosphatase activity associated with sarcoplasmic reticulum was assayed at high protein concentration (~10–20 mg/mL). Additionally, ATP-Mg^{2+} was added in the presence of 10 μM or 10 nM free Ca^{2+}, which activates or supresses the Ca^{2+} transport ATPase, respectively.

Table I: Effect of Ca^{2+} and ATP-Mg^{2+} on the Sarcoplasmic Reticular Protein Phosphatase Activity[a]

free Ca^{2+} concn	effectors	protein phosphatase act. (units/mg)	remaining act. (%)
10 nM	5 mM ATP + 10 mM Mg^{2+}	57.5×10^{-2}	100
10 μM	5 mM ATP + 10 mM Mg^{2+}	4.3×10^{-2}	7.5
10 nM	none	51.0×10^{-2}	100
10 μM	none	7.6×10^{-2}	14.9

[a] Endogenous protein phosphatase activity associated with the membranes of sarcoplasmic reticulum was assayed according to method II. The remaining activity represents the percentage of that determined in the presence of 10 nM Ca^{2+}.

Figure 1 shows that at ~10 nM free Ca^{2+} the added phosphorylated troponin can be dephosphorylated, whereas the reaction is inhibited by 10 μM Ca^{2+}. The dephosphorylation starts immediately if the free Ca^{2+} concentration is reduced by addition of an excess of EGTA (arrow, Figure 1).

Figure 2 shows that neither the removal of Ca^{2+} from troponin (A) nor the presence of AMP, (B) ATP-Mg^{2+}, (C) or additionally added purified phosphorylase kinase (D) influences the dephosphorylation rate of phosphorylated troponin by partially purified protein phosphatase (see Experimental Procedure). However, it can be seen in Table I that 10 μM Ca^{2+} in the presence of ATP-Mg^{2+} inhibits to ~92% the sarcoplasmic reticular phosphatase.

Under these assay conditions, the Ca^{2+}-induced phosphatase inhibition is due to an increase of the K_m' value for phos-

Table II: Effect of ATP-Mg^{2+} and the Catalytic Subunit of cAMP-Dependent Protein Kinase on the Ca^{2+}-Dependent Regulation of Protein Phosphatase Activity[a]

enzymes	treatment	protein phosphatase act. (units/mg) 10 nM Ca^{2+}	protein phosphatase act. (units/mg) 10 μM Ca^{2+}	inhibition (%)
sarcoplasmic reticulum with ATP-Mg^{2+} step		6.52×10^{-2}	1.70×10^{-2}	74
sarcoplasmic reticulum without ATP-Mg^{2+} step	ATP-Mg^{2+}	5.77×10^{-2}	5.79×10^{-2}	0
sarcoplasmic reticulum without ATP-Mg^{2+}	catalytic subunit, ATP-Mg^{2+}	5.90×10^{-2}	1.11×10^{-2}	87
partial purified protein phosphatase		6.65	6.92	0
partial purified protein phosphatase	catalytic subunit, ATP-Mg^{2+}	6.65	7.03	0
dissociated protein phosphatase		2.3	2.3	0
dissociated protein phosphatase	catalytic subunit, ATP-Mg^{2+}	2.3	2.4	0

[a] Vesicles of the sarcoplasmic reticulum were prepared with and without the ATP-Mg^{2+} step. The endogenous protein phosphatase activity of both membrane preparations was assayed with holotroponin according to method II. Vesicles without ATP-Mg^{2+} treatment were incubated with 15 μg/mL catalytic subunit of the cAMP-dependent protein kinase for 10 min in the presence of 5 mM ATP and 10 mM Mg^{2+}. The protein phosphatase activity was then assayed in the presence of 10 μM and 10 nM free Ca^{2+}. Partial purified protein phosphatase and the endogenous protein phosphatase remaining in the supernatant of the sarcoplasmic reticulum (20 mg/mL) after centrifugation at 140000*g* for 1 h (dissociated enzyme) were preincubated with the catalytic subunit of protein kinase under the same conditions. Thereafter, the protein phosphatase activity was assayed. 0% inhibition represents the activity in the presence of 10 nM Ca^{2+}.

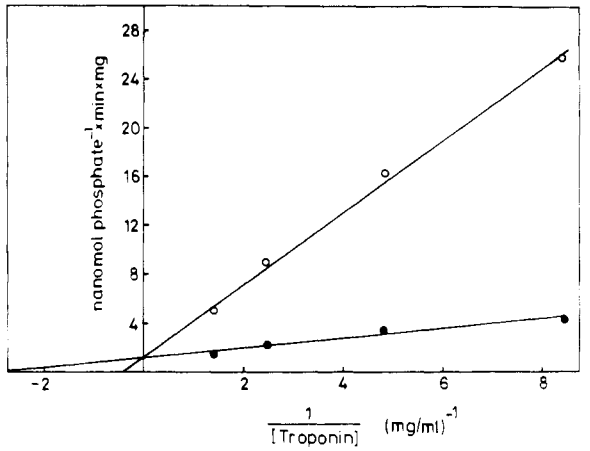

FIGURE 3: Inhibition of endogenous protein phosphatase activity by Ca^{2+}. The protein phosphatase activity was assayed at 10 μM (○) and 10 nM (●) Ca^{2+} at the troponin concentrations indicated as described by method II.

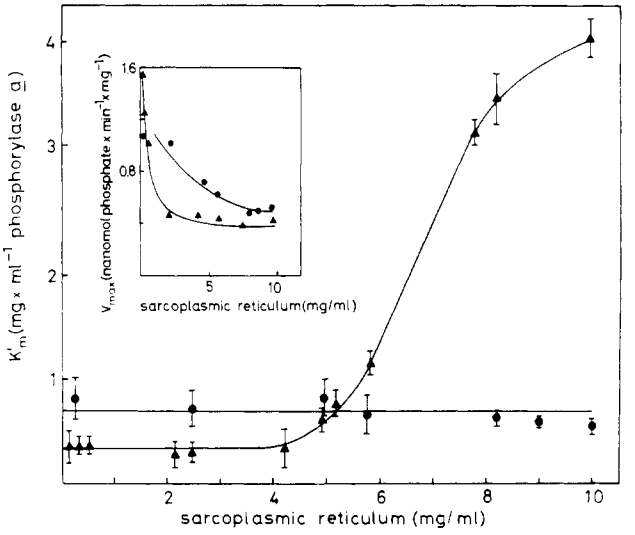

FIGURE 4: K_m' and V_{max} of endogenous protein phosphatase for phosphorylase *a* as a function of sarcoplasmic reticular protein concentration. Phosphatase activity was assayed as described by method I. The K_m' and V_{max} values in the presence of 10 μM (▲) and 10 nM (●) free Ca^{2+} were calculated by a nonlinear least-squares fit with the FORTRAN program of Cleland (1967) as modified by M. W. Kilimann and L. M. G. Heilmeyer, Jr. (unpublished experiments). The bars represent the standard deviation. The Student's *t* test indicates that the K_m' values at 0.25 and 10 mg of sarcoplasmic reticulum in the absence of free Ca^{2+} do not differ significantly ($p < 0.01$).

phorylated troponin from 0.4 to 4 mg/mL, whereas the maximal velocity does not change (Figure 3).

An increase of Ca^{2+} from 10 nM to 10 μM in the absence of ATP-Mg^{2+} inhibits the membrane-associated protein phosphatase to ~85% (Table I). It represents a control without the possible interference of the endogenous or exogenous protein kinase activity. It is in contrast to the behavior of the protein phosphatase integrated in the protein–glycogen complex. During the preparation of the sarcoplasmic reticulum, the membranes have to be incubated with 2 mM ATP, 2 mM Mg^{2+}, and 625 mM KCl (De Meis & Hasselbach, 1971) to dissociate contaminating actomyosin; the isolation of the protein–glycogen complex lacks such a step. Table II shows that the Ca^{2+}-dependent inhibition of the membrane-associated protein phosphatase activity is lost if this ATP-Mg^{2+} step is omitted during the preparation of the vesicles. The protein kinase and protein phosphatase as well as the Ca^{2+} transport ATPase activities, assayed usually after high dilution of the vesicles, are not different in both kinds of preparations. Preincubation of isolated sarcoplasmic reticulum with ATP-Mg^{2+} in the presence of 10 μM or 10 nM Ca^{2+} did not restore the Ca^{2+}-dependent phosphatase inhibition (not shown). However, inclusion of the catalytic subunit of the cyclic AMP dependent protein kinase during preincubation restored this effect. Thereafter, 87% inhibition is observed in the presence of 10 μM Ca^{2+} in comparison to 10 nM free Ca^{2+} (Table II). Incubation of partially purified protein phosphatase or of the supernatant following recentrifugation of the membranes with the catalytic subunit of the cyclic AMP dependent protein kinase under identical conditions did not produce the Ca^{2+} sensitivity of the protein phosphatase activity (Table II).

In the following, vesicles including the ATP-Mg^{2+} step were used. No ATP-Mg^{2+} was added during the protein phosphatase assays. This allowed the employment of phosphorylase *a* as substrate since no low molecular weight products are formed which would interfere in the assay.

The K_m' for phosphorylase *a* decreases approximately sevenfold upon dilution of the vesicles in the presence of 10 μM Ca^{2+} but not in the absence of Ca^{2+} (10 nM) (Figure 4). The maximal velocity increases approximately fourfold in the presence of 10 μM Ca^{2+} and two- to threefold in the presence of 10 nM Ca^{2+}; below 5.8 mg/mL sarcoplasmic reticular protein in the assay the K_m' values are not significantly different in the presence and absence of Ca^{2+} ($p < 0.01$), and

Table III: Distribution of the Protein Phosphatase Activity during Centrifugation of Sarcoplasmic Reticulum[a]

fraction	protein phosphatase act. (units/mL) with centrifugation conditions	
	10 μM Ca^{2+}	10 nM Ca^{2+}
supernatant assayed in presence of		
10 μM Ca^{2+}	1.50	3.11
10 nM Ca^{2+}	1.48	3.11
precipitate assayed in presence of		
10 μM Ca^{2+}	2.59	2.62
10 nM Ca^{2+}	4.09	4.24

[a] Sarcoplasmic reticulum (~50 mg/mL) was centrifuged in the presence of 10 μM and 10 nM free Ca^{2+} for 1 h at 140000*g*. The supernatants were carefully decanted, and the precipitates were suspended in 0.1 M KCl, yielding the original volume before centrifugation. Protein phosphatase activity was assayed in the presence of 10 μM and 10 nM free Ca^{2+} (method I).

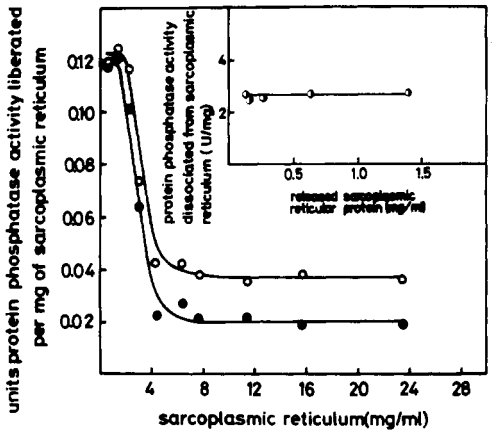

FIGURE 5: Dissociation of protein phosphatase from membranes of sarcoplasmic reticulum as a function of protein concentration. Sarcoplasmic reticulum was centrifuged at 140000*g* for 1 h after dilution with 0.1 M KCl in the presence of 10 μM (●) and 10 nM (○) free Ca^{2+} to reach the protein concentration indicated. Supernatants were decanted carefully and assayed for protein phosphatase activity as described by method I. Insert: vesicles of the sarcoplasmic reticulum (50 mg/mL) were diluted 100-fold in 0.1 M KCl, and the membranes were sedimented (see above). The supernatant was reconcentrated 10-fold by ultrafiltration over an Amicon PM 10 filter. During the reconcentration, protein concentration and protein phosphatase activity were assayed in the presence of 10 μM (●) and 10 nM (○) free Ca^{2+} as described by method I.

above this protein concentration the V_{max} values are not significantly different ($p < 0.01$).

Effect of Ca^{2+} on the Association of Protein Phosphatase with Membranes of Sarcoplasmic Reticulum. Table III shows that the supernatant following centrifugation at 140000*g* for 1 h in the presence of 10 nM Ca^{2+} contains ~100% higher protein phosphatase activity than that obtained in the presence of 10 μM Ca^{2+}. Furthermore, the solubilized protein phosphatase activity cannot be inhibited by 10 μM free Ca^{2+}, which is identical with the behavior of the partially purified enzyme (compare Figure 2). The phosphatase activity that sedimented with the membranes of sarcoplasmic reticulum kept the Ca^{2+} sensitivity; the activity assayed in the presence of this ion was ~60% of that in the absence of free Ca^{2+}.

Figure 5 shows that the amount of protein phosphatase which is released from the membranes is a function of the protein concentration; above ~5 mg/mL it remains unchanged and is approximately twice as high in the presence of 10 nM Ca^{2+} in comparison to 10 μM free Ca^{2+}. The solubilized

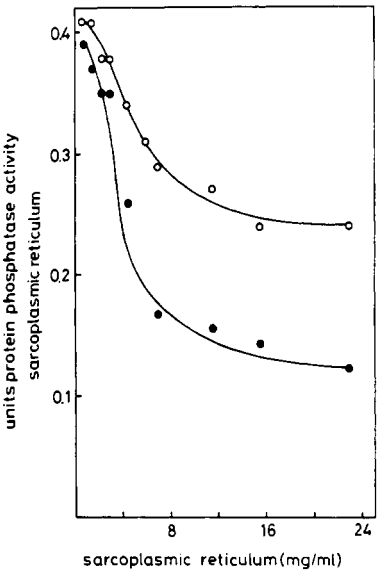

FIGURE 6: Changes in specific activity of sarcoplasmic reticular protein phosphatase as a function of protein concentration. Sarcoplasmic reticulum was diluted in 0.1 M KCl as indicated, and the protein phosphatase activity was assayed in the presence of 10 μM (●) and 10 nM (○) free Ca^{2+} as described by method I.

enzyme obtained from the supernatant after high dilution of the vesicles was reconcentrated ca. 10-fold. From the amount of released sarcoplasmic reticular protein it can be calculated that it would be equivalent to the reconcentration of the sarcoplasmic reticulum from 0.5 to 5 mg/mL (insert in Figure 5). As can be seen, the specific activity does not change.

The specific phosphatase activity assayed in the total suspension of the vesicles (not separated by centrifugation) increases upon dilution; concomitantly, the Ca^{2+} effect disappears even though part of the protein phosphatase probably remains associated with these membranes (Figure 6). Following high dilution, maximally ~30% of the total activity appears in the supernatant (compare Figures 5 and 6). In comparison with Table I and Figure 1, the phosphatase activity was assayed at a substrate concentration of ca. $(1/2)K_m'$, whereas in the latter experiment the phosphorylase *a* concentration is approximately fivefold K_m', which may be reflected in the degree of inhibition.

Discussion

The membrane-associated protein phosphatase dephosphorylates troponin phosphorylated on the T subunit (TNT-P) (see Figures 1 and 3 and Tables I and II) as well as phosphorylase *a* (Hörl et al., 1978; Hörl & Heilmeyer, 1978).

The main advantage of the substrate TNT-P is its insensitivity to the presence of low molecular weight substances like ATP–Mg^{2+} or AMP, which inhibit the phosphorylase *a* but not the troponin dephosphorylation. It excludes that breakdown products of ATP are responsible for the observed Ca^{2+}-dependent protein phosphatase inhibition. In addition, removal of Ca^{2+} from TNT does not affect the dephosphorylation of the TNT subunit in the holotroponin complex (compare Figure 2).

At high protein concentration the kinetic analysis reveals that the sarcoplasmic reticular protein phosphatase is inhibited competitively in the presence of 10 μM free Ca^{2+} (Figure 3 and 4), as measured with both substrates, phosphorylated troponin and phosphorylase *a*. On the other hand, it can be shown with phosphorylase *a* as substrate that dilution of the sarcoplasmic reticulum at 10 nM Ca^{2+} increases the V_{max} value two- to threefold at a constant K_m' value, whereas in the

presence of 10 μM Ca^{2+} both of these parameters change (compare Figure 4).

The latter probably does not represent a mixed type inhibition since both parameters do not change in parallel with the sarcoplasmic reticular protein concentration.

Only that part of the enzyme which sediments with the membranes shows the Ca^{2+}-dependent inhibition (Table III). The solubilized enzyme is insensitive to Ca^{2+}, and additionally it does not change its specific activity with protein concentration (compare insert in Figure 5).

Following sedimentation of the sarcoplasmic reticulum at a higher protein concentration than 5 mg/mL in the presence of 10 μM Ca^{2+}, a lesser amount of phosphatase is found in the supernatant than at 10 nM Ca^{2+} (Figure 5). Therefore, one might assume that in the presence of Ca^{2+} the protein phosphatase associates with the membranes and dissociates upon dilution. Consequently, at a high dilution the protein phosphatase activity is no longer inhibited at 10 μM Ca^{2+} (compare Figure 6). However, only ~30% of the total enzymatic activity is maximally released from the membranes upon high dilution (compare Figures 5 and 6), whereas from the complete Ca^{2+} insensitivity a 100% dissociation would be expected. In contrast to the sedimentation experiments, the assays of the activity (Figure 6) in the vesicle suspension were preformed in the presence of substrate, which probably influences the competitive inhibition at high protein concentration and in the presence of Ca^{2+}. Upon dilution, an increase of the total activity is observed which is probably due to an increase of V_{max} (compare Figures 4 and 6). Again at the highest dilution both the competitive and the noncompetitive inhibitions disappear.

Recently, two soluble heat-stable inhibitors, I and II, of the protein phosphatase have been characterized (Huang & Glinsmann, 1975, 1976a,b; Nimmo & Cohen, 1978a,b). Inhibitor I inhibits only in the phosphorylated form. Both inhibitors show a noncompetitive inhibition with respect to phosphorylase *a* (Huang & Glinsmann, 1976a,b; Nimmo & Cohen, 1978a,b).

Heat treatment of the sarcoplasmic reticulum reveals also the presence of a heat-stable inhibitor in these membranes. However, this inhibitor does not confer Ca^{2+} sensitivity to the phosphatase activity (M. Varsányi and L. M. G. Heilmeyer, Jr., unpublished experiments). Therefore, this inhibitor was not yet characterized for its identity with the known heat-stable inhibitors.

In one respect the inhibition caused by inhibitor I is similar to the membrane-associated phosphatase inhibition. Similar to this latter polypeptide, an inhibition was only observed after preincubation with the catalytic subunit of the cAMP-dependent protein kinase in the presence of ATP–Mg^{2+} when a phosphorylation reaction can occur. Such a phosphorylation is catalyzed neither by phosphorylase kinase (not shown) nor by Ca^{2+}-dependent protein kinase present in the membranes of the sarcoplasmic reticulum (Table II). The phosphorylation of proteins different from the Ca^{2+} transport ATPase has been reported (Heilmann et al., 1977). Phosphorylation may occur during the preparation of the vesicles, including the ATP–Mg^{2+} step. Presumably, with further purification the protein kinase activity is lost and must be readded to restore the Ca^{2+} sensitivity, i.e., probably the phosphorylated state. The competitive-type inhibition of this membrane-associated phosphatase is different from the type exerted by the phosphorylated, heat-stable inhibitor I, which is, like inhibitor II, a noncompetitive inhibitor (Antoniw et al., 1977; Huang & Glinsmann, 1976b).

The chelating properties of ATP or PP_i lead to an inhibition of several kinds of protein phosphatases due to formation of an apoenzyme which lacks probably Mn^{2+} (Hsiao et al., 1978). If ATP was used in the assay of the membrane-associated protein phosphatase, Mg^{2+} was present which prevents this apoenzyme formation (Hsiao et al., 1978). In addition, ATP–Mg^{2+} has also no effect on the partially purified enzyme, and finally for the Ca^{2+} effect, no ATP–Mg^{2+} itself is needed. Therefore, the observed inhibition induced by Ca^{2+} can probably not be due to apoenzyme formation.

Even though no effect of Ca^{2+} on the solubilized protein phosphatase or the partially purified enzyme is observed, this metal ion may act via the phosphatase or the membranes; vice versa, either the phosphatase or the membranes must bind Ca^{2+}. Prominently, the Ca^{2+} transport ATPase binds Ca^{2+} in sarcoplasmic reticulum (Ikemoto, 1974, 1975), but other components like the Ca^{2+}-dependent protein kinase or the high-affinity Ca^{2+} binding protein also bind Ca^{2+} to a lower extent (Hörl et al., 1978; Ostwald & MacLennan, 1974). The inhibition of the Ca^{2+} transport ATPase by the protein phosphatase may hint to an interaction of these two proteins (Hörl & Heilmeyer, 1978). However, the ATPase need not to be active since the Ca^{2+}-induced protein phosphatase inhibition is also observed in the absence of ATP–Mg^{2+}. Therefore, the effect of the phosphatase on this ATPase may also be indirectly through other components of the sarcoplasmic reticulum.

It is known that a heat-stable Ca^{2+} binding protein, the δ subunit of phosphorylase kinase, confers Ca^{2+} sensitivity to a variety of protein kinases (Dabrowska et al., 1977; Yagi et al., 1978; Cohen et al., 1978). It was tested that the Ca^{2+} modulator protein which was isolated from phosphorylase kinase did not confer Ca^{2+} sensitivity to the partially purified protein phosphatase (M. Varsányi and L. M. G. Heilmeyer, Jr., unpublished experiments).

Detwiler et al. (1977) have shown that dilution of purified phosphorylase phosphatase from 440 to 22 units/mL changes the K_m' and V_{max} values. The phosphatase concentration in the sarcoplasmic reticulum ranges approximately from 0.1 to 5 units/mL. It was demonstrated that at this low protein phosphatase concentration the specific activity does not change, and therefore a self-association of the enzyme cannot be responsible for the observed changes in the K_m' and V_{max} values (insert in Figure 5).

In comparison to the protein–glycogen complex, the Ca^{2+}-dependent inhibition of the membrane-associated phosphatase was observed with a ca. 100-fold lower phosphatase concentration. Therefore, it is difficult to compare directly the behavior of the phosphatase in the protein–glycogen complex with the effect shown here. However, qualitatively the same Ca^{2+}-dependent inhibition is observed here and in the protein–glycogen complex.

Acknowledgments

We thank Gabriele Brinkmann, Sibylle Rigler, and Ulrich Siemen for the excellent technical assistance.

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