Biochemistry

 Volume 17, Number 5 March 7, 1978

Evidence for the Participation of a Ca^{2+}-Dependent Protein Kinase and a Protein Phosphatase in the Regulation of the Ca^{2+} Transport ATPase of the Sarcoplasmic Reticulum. 1. Effect of Inhibitors of the Ca^{2+}-Dependent Protein Kinase and Protein Phosphatase†

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ABSTRACT: A Ca^{2+}-dependent protein kinase which is associated with the sarcoplasmic reticulum converts phosphorylase *b* to *a* and exhibits a pH 6.8/8.6 activity ratio of ca. 0.5. While catalyzing this interconversion the Ca^{2+} transport ATPase activity assayed in the presence of 4 mM oxalate is reduced by 30%. Half-maximal inhibition of phosphorylase *b* to *a* conversion catalyzed by the membrane bound kinase requires a ca. threefold higher anti-phosphorylase kinase concentration than that catalyzed by purified soluble phosphorylase kinase. A further ca. twofold increase of the anti-phosphorylase kinase concentration half-maximally inhibits the ATPase activities assayed in the absence or presence of 4 mM oxalate. The antibodies do not cross-react with any of the other main components of the sarcoplasmic reticulum. The Ca^{2+} uptake rate is reduced by anti-phosphorylase kinase to the same extent as the ATPase activity. Hydroxylamine inhibits as a function of its concentration phosphorylase kinase and Ca^{2+} transport ATPase activity as well as phosphoprotein formation. Oxalate or fluoride maximally stimulates the Ca^{2+} transport ATPase activity at concentrations which cause a ca. 80% inhibition of the protein phosphatase activity. Hydroxylamine inhibits the Ca^{2+} transport ATPase activity probably mediated by an inhibition of the Ca^{2+}-dependent protein kinase activity. Antagonistically oxalate stimulates the Ca^{2+} transport ATPase which may be caused by an inhibition of a protein phosphatase activity. It is concluded that the Ca^{2+} transport ATPase activity is regulated by the balance of the Ca^{2+}-dependent protein kinase and protein phosphatase activity.

Studies with purified fluorescein isothiocyanate labeled anti-phosphorylase kinase indicated that the phosphorylase kinase antigen is present in the sarcoplasm and in addition in muscle membranes (Hörl et al., 1975a,b). A low amount of this kinase and additionally phosphorylase phosphatase activity could not be separated by sucrose gradient centrifugation from the sarcoplasmic reticulum isolated from rabbit skeletal muscle. However, no phosphorylase *b* is found in these purified membranes (Hörl et al., 1975b; Jennissen et al., 1976). The inhibition of the oxalate stimulated Ca^{2+} transport (Hörl and Heilmeyer, 1976, 1977) by anti-phosphorylase kinase suggested that the phosphorylase kinase is involved in the regulation of this ATPase. Later Schwartz et al. (1976) showed that addition of phosphorylase kinase to skeletal sarcoplasmic reticulum isolated from various muscles could stimulate in some preparations Ca^{2+} uptake as well as Ca^{2+} transport ATPase activity. In agreement with Katz et al. (1973), no effects of cAMP and cAMP dependent protein kinase were observed on the phosphorylation of fast twitch skeletal muscle sarcoplasmic reticulum (Schwartz et al., 1976).

Free Ca^{2+} (10^{-6} M) activates the transport ATPase (Hasselbach and Makinose 1961; Ebashi and Lippmann, 1962; Martonosi and Feretos, 1964) and also saturates phosphorylase kinase with this cation (Brostrom et al., 1971; Kilimann and Heilmeyer, 1977). Accumulation of Ca^{2+} ions inside the vesicles in millimolar concentrations inhibits the ATPase activity (Hasselbach and Makinose, 1961, 1963). Consequently, effects of anions such as oxalate which activate the transport ATPase activity ca. twofold have been interpreted as intravesicular Ca^{2+} precipitating agents. By this way the intravesicular Ca^{2+} concentration is lowered to noninhibitory levels (Hasselbach and Makinose, 1963; Makinose and Hasselbach, 1965).

A phosphorylated form of the ATPase was concluded to be an intermediate of the catalytic cycle. After denaturation with trichloroacetic acid, a phosphopeptide could be isolated which contains an aspartyl phosphate residue (Post and Kume, 1973). This energy-rich phosphate easily reacted with hydroxylamine to the corresponding very stable hydroxamic acid. In contrast,

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§ Supported by Grant No. Je 84/3 of the Deutsche Forschungsgemeinschaft.

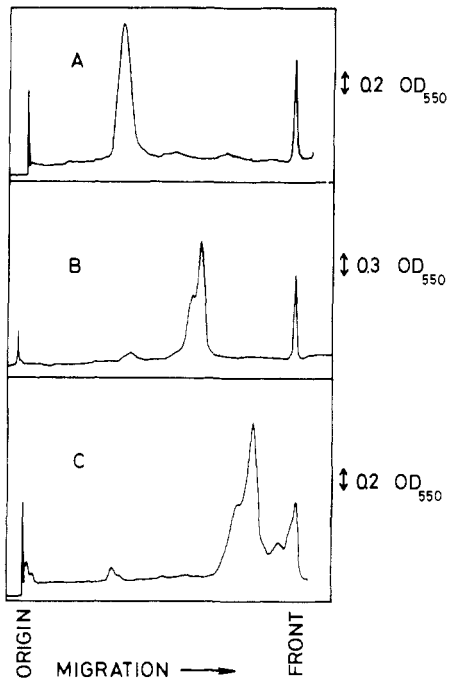

FIGURE 1: $NaDodSO_4$ gel electrophoresis of isolated vesicular components. Fractions obtained during preparative isolation of the main components of sarcoplasmic reticulum were subjected to polyacrylamide (5%) electrophoresis in presence of $NaDodSO_4$. From ca. 20 mg of purified vesicles ca. 15 mg of ATPase, 0.5 mg of protein with a molecular weight in the range of calsequestrin, and 0.6 mg of acidic proteins were obtained. (A) Shows 2.8 μg of ATPase (mol wt 100 000); (B) 7 μg of calsequestrin (mol wt 50 000–54 000); and (C) 3 μg of acidic proteins (mol wt 30 000–34 000).

the native ATPase is reversibly inhibited by this amine (Inesi et al., 1970). It was concluded that hydroxylamine interferes with another, until now unidentified, step of the Ca^{2+} transport ATPase.

This publication will present evidence that anions activate the Ca^{2+} transport ATPase indirectly by means of inhibition of a protein phosphatase. A Ca^{2+}-dependent protein kinase can be inhibited with anti-phosphorylase kinase which results in a parallel reduction of the Ca^{2+} transport ATPase activity. The reversible inhibition of the Ca^{2+} transport ATPase by hydroxylamine can also be interpreted as an inhibition of the Ca^{2+}-dependent protein kinase. Therefore, a Ca^{2+}-dependent protein kinase and protein phosphatase may regulate the Ca^{2+} transport ATPase activity.

Materials and Methods

The Ca^{2+} transport ATPase of the sarcoplasmic reticulum was assayed according to Hasselbach (1966) with the following modifications: the reaction mixture (30 °C) contained 100 mM KCl, 50 mM imidazole hydrochloride (pH 7.0), 5 mM $MgCl_2$, 1.5 mM EGTA[1] and vesicles in a concentration of ca. 0.1 mg/mL. The reaction was started by addition of ATP (final concentration 5 mM). After 2 and 4 min, aliquots of 0.2 mL were removed and added to 0.4 mL of $NaDodSO_4$ (10%). After 5 min, Ca^{2+} was added (final concentration, 1.3 mM) and the reaction was stopped as described at 5 min 30 s, 6 min, and 6 min 30 s. After 7 min, oxalate was added (final concentration, 4 mM) and samples were removed and treated as before after 7 min 30 s, 8 min, 8 min 30 s, 9 min, and 10 min. Inorganic phosphate was determined on an autoanalyzer analogously to the assay of phosphorylase activity. For calculation of the Ca^{2+} transport ATPase activity, the basal ATPase activity measured before Ca^{2+} addition is subtracted. The oxalate/Ca^{2+} activity ratio represents the quotient of the ATPase activities after and before oxalate addition. [γ-^{32}P]-ATP was synthesized according to Glynn and Chappel (1964). Ca^{2+} and oxalate uptake in sarcoplasmic reticulum was measured in aliquots (ca. 1 mg of protein) filtered through Millipore (filter, type EG, 0.2 μm). Phosphorylation of the Ca^{2+} transport ATPase was carried out according to Makinose (1969).

Sarcoplasmic reticulum was prepared as described by De Meis and Hasselbach (1971). Phosphorylase *b* was crystallized under the conditions described by Fischer and Krebs (1958). Phosphorylase kinase was isolated following the method of Cohen (1973) modified according to Jennissen and Heilmeyer (1975). The subunits of phosphorylase kinase were purified according to Jennissen et al. (1976). Phosphorylase *b* and phosphorylase kinase were determined on an autoanalyzer (Haschke-and Heilmeyer, 1972; Jennissen and Heilmeyer, 1974). Phosphorylase phosphatase was assayed according to Haschke et al. (1970). Protein kinase was determined as described by Reimann et al. (1971).

Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was carried out according to Weber and Osborn (1969). Protein was determined by the Lowry method (Lowry et al., 1951). Reagents used were analytical grade.

Isolation of Vesicular Components. Sixty milligrams of lyophilized vesicles was taken up in 4 mL of 50 mM Tris-HCl, 1 mM DTE, 20% sucrose, pH 7.0, and centrifuged at 11 000*g* for 10 min which was repeated three times. Finally the pellet was suspended in 1.0 mL of buffer to which 20 μL of 14 M mercaptoethanol and 0.1 mL of 10% $NaDodSO_4$ were added. This mixture was heated to 100 °C for 10 min and applied to a column (2 × 100 cm) containing Ultrogel ACA 44 (LKB, Uppsala) equilibrated with 10 mM sodium phosphate, 0.1% $NaDodSO_4$, 1 mM DTE, 0.05% azide, 10^{-4} M $PhCH_2SO_2F$, pH 6.4. The column was eluted at a rate of 20 mL/h. By this procedure, a crude separation into the 100 000, 50 000–60 000, and 30 000 molecular weight components was obtained. These fractions were pooled and concentrated by ultrafiltration on an Amicon PM 10 filter. The material was applied to a preparative electrophoresis system (Colora Ultraphor). The electrophoretic separation of the polypeptides was obtained in the presence of $NaDodSO_4$ in a discontinuous buffer system as described by Neville (1971) on 7% slab gels, 140 × 75 × 40 mm. The sample (containing ca. 5 mg of polypeptide in 5 mL) was concentrated on a 3% sample gel (140 × 75 × 10 mm). For the concentration a constant current of 30 mA was applied for ca. 1 h. The current was then increased to 90 mA for the separation. The separated polypeptides were eluted at a rate of 55 mL/h with the same buffer previously used at the anode compartment. The fractions containing the pure polypeptides were pooled and concentrated in an Amicon cell employing a PM 10 filter. Fractions obtained are shown in Figure 1.

Immunization of Sheep. Male Texel sheep (castrated) (Fa. Hoechst AG, Frankfurt) of 36–47 kg were immunized by intramuscular injection into the hind legs of 2.0 mL antigen solution (phosphorylase kinase, 40 mg, purified according to De Lang et al. (1968) in 10 mM sodium β-glycerophosphate, 4 mM mercaptoethanol, pH 7.0), emulsified with 2.0 mL of complete Freunds adjuvant (Bacto adjuvants, Difco) (Clark and Mauer, 1969). Primary immunization was achieved by injecting the above emulsion into alternating legs at the days:

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DTE, dithioerythritol; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; cAMP, cyclic adenosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

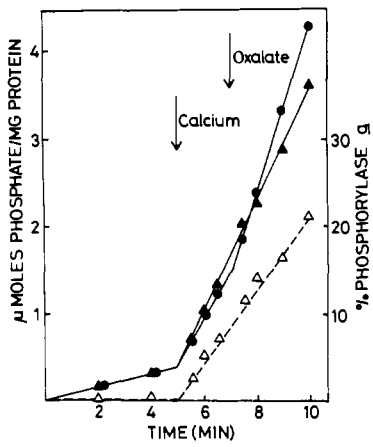

FIGURE 2: Influence of phosphorylase *b* on the Ca^{2+} transport ATPase. ATPase activity was assayed as described in Materials and Methods in absence (—●—) and presence (—▲—) of 4.5 mg/mL phosphorylase *b*. The vesicular protein concentration for determination of the ATPase activity was 0.15 mg/mL, for determination of the rate of phosphorylase *b* to *a* conversion (—△—) 0.65 mg/mL. At zero time, the reaction was started by addition of 5 mM ATP. At the time points indicated by arrows, Ca^{2+} was added to a final free metal ion concentration of 1.4×10^{-6} M (EGTA 1.5 mM) and oxalate of 4 mM. For determination of the amount of phosphorylase *a* formed, aliquots were removed, 100-fold diluted in stop buffer, and assayed for phosphorylase *a* activity as referred to in Materials and Methods.

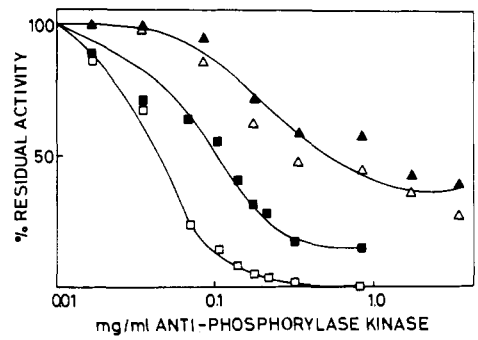

FIGURE 3: Inhibition of purified phosphorylase kinase, vesicular Ca^{2+}-dependent protein kinase, and Ca^{2+} transport ATPase by antiphosphorylase kinase. The percent residual enzyme activity is plotted vs. the logarithm of the amount of antibody added. One hundred percent activity represents the activity without added antibody. Phosphorylase kinase activity (—□—) and membrane bound protein kinase activity (—■—) were assayed as described in Materials and Methods. Transport ATPase activity in presence of Ca^{2+} only (—△—) or additionally in presence of 5 mM oxalate (—▲—) was measured as described in Materials and Methods. Antibody as indicated was added to 3 U/mL phosphorylase kinase (7.6 U/mg) or to 0.031 U/mL membrane-bound protein kinase (0.4 U/mg) or to 0.1 mg/mL vesicular protein. The mixture was incubated for 10 min at 20 °C prior to the activity assay.

1, 8, and 15. Booster shots were given at the days: 46, 50, and 74. Blood samples were taken on the days of injection and the sheep were bled for collection of anti-serum on days 57, 63, 91, 98, and 105 (ca. 300 mL of blood per day). Anti-phosphorylase kinase activity was first detected on day 15.

Purification of Antibodies against Phosphorylase Kinase. One milliliter of phosphorylase kinase (8.8 mg/mL in 50 mM sodium β-glycerophosphate, pH 7.0, 20 mM mercaptoethanol) was added to 9.0 mL anti-phosphorylase kinase (γ_1-globulin fraction, 40 mg/mL, purified according to Stylos et al., 1970) in 50 mM sodium β-glycerophosphate, pH 7.0. The precipitin reaction was allowed to proceed for 15 min at ca. 20 °C. The mixture was centrifuged at room temperature for 10 min at 3000*g*. The pellet was washed three times with 6 mL of 50 mM sodium β-glycerophosphate, pH 7.0. The final product was solubilized in 1 mM HCl. To this solution 0.01 volume of 3.5 M ammonium sulfate, pH 3.0, was added. The protein precipitate was removed by centrifugation. The supernatant contained the purified antibody which was employed.

Double Diffusion System (Ouchterlony, 1949). (a) Native Enzyme. The diffusion was carried out in 0.8% agarose (Serva, analytical grade), 50 mM sodium β-glycerophosphate, 0.1% azide, pH 7.0. Gel (2.5 mL) was applied to a microscopic slide (7.5 × 2.5 cm). The well diameter was 3.5 mm and the distance to the center well 8 mm. After 48 h at 20 °C the slide was washed with 50 mM sodium β-glycerophosphate, pH 7.0, for 10 h followed by a 1-h wash in water.

(b) $NaDodSO_4$ Denatured Enzyme. The $NaDodSO_4$ denatured polypeptides were dialyzed against 0.1 M glycine, 0.038 M Tris (Clark and Freeman, 1967), to which 1% Triton X-100 was added. The diffusion was carried out in the same buffer in 0.8% agarose. After a diffusion time of 48 h at 20 °C, the gel was washed in the same buffer for 5–6 h followed by a 10-h wash in 0.1 M NaCl and 1-h wash in water. The gel slides were air dried after covering the surface with a wet filter paper. For staining, a solution of 0.5% Amido black (Serva, Heidelberg) in methanol/acetic acid (9:1) was used. Destaining was carried out in the same solvent.

Results

The influence of Ca^{2+} on the ATPase and on the protein kinase which is associated with the sarcoplasmic reticulum is shown in Figure 2. In the presence of EGTA (free Ca^{2+} ca. 10^{-8} M), no conversion of phosphorylase *b* to *a* is observed and a basal ATPase activity is measured.

Phosphorylase can only be interconverted following addition of Ca^{2+} which yields a free metal ion concentration of ca. 10^{-6} M. Simultaneously the ATPase activity increases three- to fivefold; this activity is referred to as Ca^{2+} transport ATPase or extra ATPase (Hasselbach and Makinose, 1963). In absence of phosphorylase *b*, 4 mM oxalate additionally enhances its activity ca. twofold. The presence of phosphorylase *b* prevents this oxalate stimulation of the ATPase activity but this anion has no effect on the *b* to *a* conversion (Figure 2). About 20% of the added phosphorylase *b* is converted to the *a* form. As a control, addition of an equivalent amount of serum albumin has no effect on the ATPase activity. The pH 6.8/8.2 activity ratio of the membrane associated kinase is ca. 0.5.

The influence of this Ca^{2+}-dependent protein kinase on the Ca^{2+} transport ATPase can be demonstrated by titration of the sarcoplasmic reticulum vesicles with antibodies against phosphorylase kinase. Figure 3 shows that the membrane-bound, Ca^{2+}-dependent protein kinase (assayed as phosphorylase kinase) requires a two- to threefold higher antibody concentration for half-maximal inhibition than soluble, purified phosphorylase kinase. Even at the highest antibody concentration employed, the membrane-bound kinase activity is not completely inhibited in contrast to purified homogeneous phosphorylase kinase. The residual activity is not due to AMP formation which could allosterically activate phosphorylase *b*. Routinely this latter activity was determined in control experiments and was subtracted (see Jennissen and Heilmeyer, 1974). These antibodies also inhibit the ATPase activities in presence or absence of oxalate, respectively (Figure 3). The antibody concentration which causes half maximal inhibition is two- to fourfold higher than that which produces half-maximal inhibition of the membrane-bound protein kinase activity. The ATPase activities cannot be completely inhibited by anti-phosphorylase kinase, the residual activities amount to 35–40% of those determined without antibody.

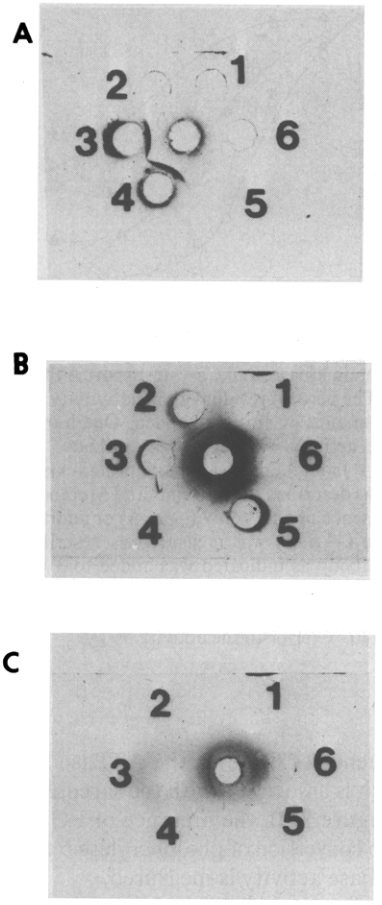

FIGURE 4: (A) Double diffusion of anti-phosphorylase kinase against native phosphorylase kinase. The center well contained 20 μL of anti-phosphorylase kinase (40 mg/mL) and wells 3 and 4, 32 μg phosphorylase kinase in 20 μL. Both proteins were dialyzed against the gel buffer. All other conditions are described in Materials and Methods. (B) Double diffusion of anti-phosphorylase kinase against the subunits of phosphorylase kinase. The center well contained 20 μL of anti-phosphorylase kinase (40 mg/mL) dialyzed against the gel buffer. (Well 1) Contained 10 μL of subunit α (0.11 mg/mL); (well 2) 10 μL of subunit β (0.13 mg/mL); and (well 3) 10 μL of subunit γ (0.15 mg/mL); (well 5) contained a mixture of all three subunits in the same amounts as applied separately in a total volume of 15 μL. All other conditions are described in Materials and Methods. (C) Double diffusion of actin, the γ subunit of phosphorylase kinase, and vesicular proteins against anti-phosphorylase kinase. The center well contained 20 μL of anti-phosphorylase kinase (40 mg/mL). (Well 1) Contained actin (28 μg/20 μL); (well 3) subunit γ (30 μg/20 μL); and (well 5) vesicular proteins (64 μg/20 μL). All proteins (B, C; wells 1–5) were denatured in $NaDodSO_4$ and dialyzed against the gel buffer containing Triton X-100 (see Materials and Methods).

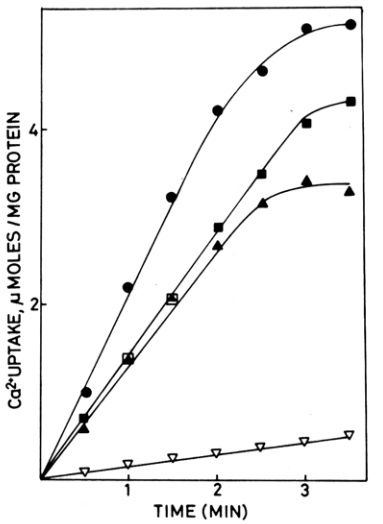

FIGURE 5: Effect of anti-phosphorylase kinase and phosphorylase *b* on the Ca^{2+} uptake rate of the sarcoplasmic reticulum. To 0.15 mg/mL vesicular protein was added 3.0 mg/mL purified γ_1-globulin fraction (see Materials and Methods) (—▲—). Phosphorylase *b* (—■—) was present at a concentration of 4.5 mg/mL. Leaky vesicles (—▽—) were obtained by adjustment of the pH to 7.5. Ca^{2+} uptake was measured without addition (—●—) as referred to in Materials and Methods.

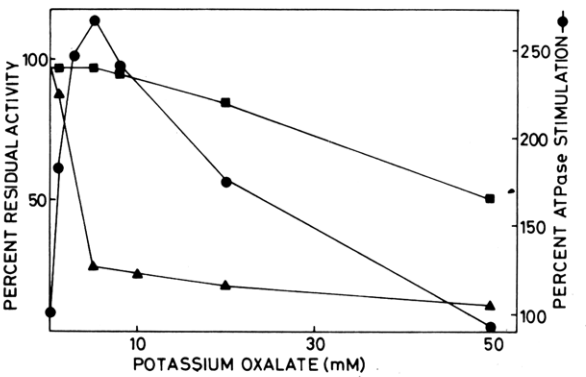

FIGURE 6: Effect of potassium oxalate on the activites of phosphorylase kinase (—■—), phosphorylase phosphatase (—▲—), and Ca^{2+} transport ATPase (—●—). The specific activities of these three enzymes were 7.95, 0.015, and 0.74 U/mg, respectively, which represent 100%. Enzymatic activities were assayed as described in Materials and Methods, except for phosphorylase kinase activity which was determined in presence of half the equivalent Mg^{2+} concentration of the added oxalate.

The antibody employed was purified by immunoprecipitation. It yields one precipitin line in the double-diffusion test with native purified soluble phosphorylase kinase (Figure 4A). When the purified isolated subunits α, β, and γ are used as antigens, each of these polypeptides yields a precipitin line (Figure 4B). In the case of the α subunit two precipitin lines were obtained. Under identical conditions none of the main vesicular components which were purified as $NaDodSO_4$–protein complexes by preparative gel electrophoresis (see Materials and Methods) and which do not contain the polypeptides corresponding to α and β of phosphorylase kinase showed a cross-reaction with anti-phosphorylase kinase. Also isolated rabbit muscle actin did not cross-react with these antibodies (Figure 4C).

An antibody concentration which reduces the Ca^{2+} uptake rate as measured in the presence of oxalate by ca. 44% (Figure 5) inhibits the oxalate stimulated ATPase activity to the same extent. Phosphorylase *b* addition reduces the Ca^{2+} uptake rate by ca. 38% (Figure 5) and the Ca^{2+} transport ATPase activity to ca. 30%. The addition of antibody reduces the maximal Ca^{2+} uptake by ca. 34% and phosphorylase *b* by ca. 20%. Equivalent amounts of [^{14}C]oxalate are accumulated (not shown).

Figure 6 shows that 5 mM oxalate maximally stimulates the Ca^{2+} transport ATPase, whereas it does not influence phosphorylase kinase activity; in contrast, phosphorylase phosphatase activity is inhibited to ca. 80%. The described effects are not due to the increase of ionic strength; equimolar concentrations of NaCl have no influence on these three activities (not shown).

Analogously, fluoride inhibits phosphorylase phosphatase activity at a concentration of 20 mM to ca. 80% (Figure 7), whereas it does not influence phosphorylase kinase activity. Again an optimal stimulation of the Ca^{2+} transport ATPase activity is obtained at that fluoride concentration which inhibits the protein phosphatase activity to ca. 80%. Figure 8 demonstrates that a reduction of the purified phosphorylase kinase activity by hydroxylamine can be correlated with an inhibition

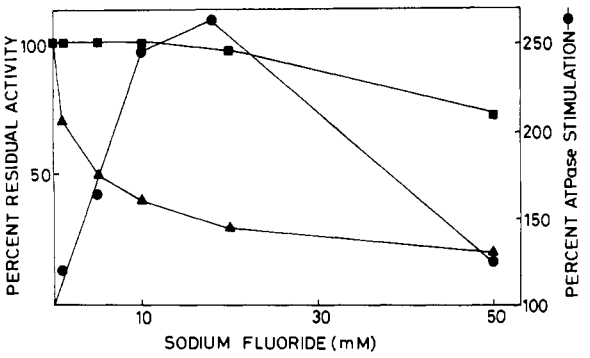

FIGURE 7: Effect of sodium fluoride on the activities of phosphorylase kinase (—■—), phosphorylase phosphatase (—▲—), and Ca^{2+} transport ATPase (—●—). The specific activities of these three enzymes were 7.95, 0.015, and 0.74 U/mg, respectively, which represent 100%. Enzymatic activities were assayed in presence of fluoride as described in Materials and Methods.

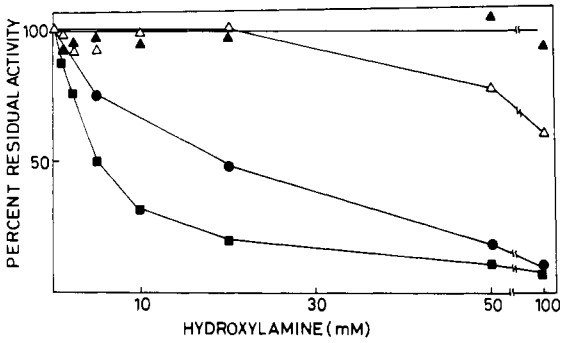

FIGURE 8: Effect of hydroxylamine on the activities of purified phosphorylase kinase (—■—), purified phosphorylase phosphatase in presence (—▲—) and absence (—△—) of 10^{-4} M Mn^{2+}, and Ca^{2+} transport ATPase (—●—). The specific activities of these three enzymes were 7.95, 0.015, and 0.74 U/mg, respectively, which represent 100%. Enzymatic activities were assayed in presence of hydroxylamine as described in Materials and Methods.

of the Ca^{2+} transport ATPase activity. The maximal inhibition of phosphorylase kinase by hydroxylamine varies with the preparation of the enzyme between 50% and 90%. Similarly, the hydroxylamine inhibition of the ATPase activity decreases with aging of the vesicles (compare Hörl and Heilmeyer, 1978). Concentrations lower than 100 mM hydroxylamine have no effect on the protein phosphatase activity in presence of 10^{-4} M Mn^{2+}; in absence of this cation, a ca. 40% decrease is observed (Figure 8).

Addition of Ca^{2+} stimulates the Ca^{2+} transport ATPase as well as phosphoprotein formation (Figure 9). Upon addition of hydroxylamine, the amount of phosphoprotein very rapidly decreases and simultaneously the ATPase activity is lowered to the rate of the basal activity. Removal of hydroxylamine by sedimentation of the vesicles and resuspension in fresh buffer allows a complete reactivation of the Ca^{2+} transport ATPase activity with a concomitant high level of phosphoprotein formation (ca. 0.25 mol of P_i incorporated per 10^5 g of protein). The data indicate that the Ca^{2+} transport ATPase activity depends on the activity ratio of the Ca^{2+}-dependent protein kinase and protein phosphatase. Therefore, a partial inhibition of the Ca^{2+} transport ATPase activity mediated by the Ca^{2+}-dependent protein kinase with hydroxylamine should be possible to be reversed by an inhibition of the protein phosphatase with oxalate. To avoid complication of such an experiment by inhibition of the ATPase due to intravesicular accumulation of Ca^{2+}, the vesicles were adjusted to pH 7.5 which destroys the Ca^{2+} accumulation capability (see Figure

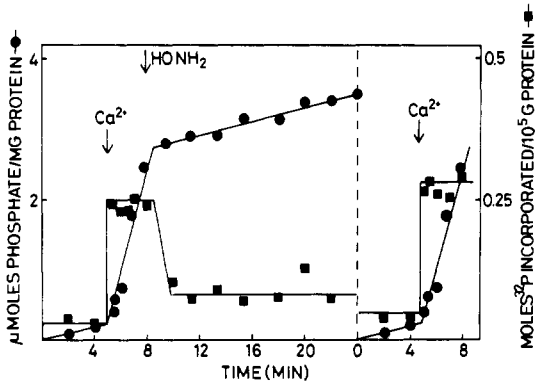

FIGURE 9: Influence of hydroxylamine on Ca^{2+} transport ATPase activity and E~P formation. ATPase activity (—●—) and E~P formation (—■—) were determined as described in Materials and Methods. Hydroxylamine (0.1 M) was added (arrow). Following centrifugation at 12 000*g* for 10 min, the vesicles were resuspended in the test incubation mixture (dotted line). The reaction was started again as described above.

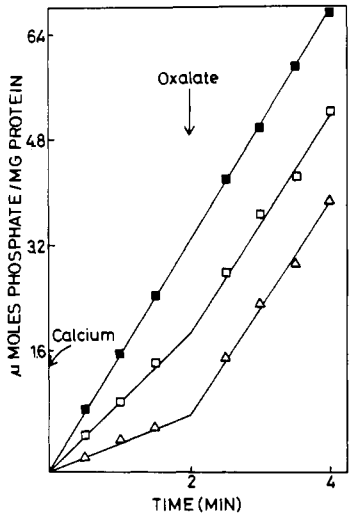

FIGURE 10: Effect of 50 mM (—□—) and 100 mM (—△—) hydroxylamine together with 4 mM oxalate on the Ca^{2+} transport ATPase activity of leaky vesicles. Vesicles of the sarcoplasmic reticulum were made leaky by adjustment of the pH to 7.5 (—■—) (compare Figure 4). ATPase activities in presence of Ca^{2+} and oxalate (arrows) were determined as described in Materials and Methods.

5). The behavior of the employed preparation is shown in Figure 5. Probably due to an increase of the Ca^{2+}-dependent protein kinase in relation to the protein phosphatase activity these opened vesicles show no stimulation of the transport ATPase activity by oxalate (Figure 10). Nevertheless, a stimulation by oxalate can be produced if the protein kinase activity is partially inhibited with hydroxylamine. Under these conditions the phosphatase inhibitor, oxalate, stimulates the Ca^{2+} transport ATPase activity maximally fourfold (Figure 10).

Discussion

Isolated vesicles of the sarcoplasmic reticulum from rabbit skeletal muscle contain a protein kinase which in presence of Ca^{2+} ions converts phosphorylase *b* to *a* as known for soluble purified phosphorylase kinase (Brostrom et al., 1971; Heilmeyer et al., 1970) (compare Figure 2). The pH 6.8/8.2 activity ratio of the membrane bound enzyme differs from that of nonactivated purified phosphorylase kinase which shows a ratio of 0.05. A similar observation was made earlier by

Hammermeister et al. (1965) who demonstrated that in heart muscle an "insoluble fraction" of this enzyme exhibited a higher pH 6.8/8.2 ratio of 0.53 than the soluble enzyme. An increased pH 6.8/8.2 activity ratio can be caused by either phosphorylation or partial proteolytic degradation of the α and β subunits of phosphorylase kinase (Krebs et al., 1964; Cohen, 1973). Alternatively this enhanced ratio might be due to an altered structure which is indicated by the decreased sensitivity of this membrane associated protein kinase to anti-phosphorylase kinase (compare Figure 3). Furthermore, sarcoplasmic reticulum isolated from I-strain mice which lacks completely soluble phosphorylase kinase (Cohen et al., 1976) also contains a membrane associated Ca^{2+}-dependent protein kinase. In presence of Ca^{2+} ions this kinase is able to convert phosphorylase *b* to *a* (Varsànyi and Heilmeyer, in preparation) which demonstrates the existence of a genetically and therefore very probably structurally different Ca^{2+}-dependent protein kinase from phsophorylase kinase in these membranes.

While catalyzing the *b* to *a* interconversion, the oxalate-stimulated Ca^{2+} transport ATPase is reduced (Figure 2). An explanation could be that phosphorylase *b* competes on this kinase with an alternate substrate, the Ca^{2+} transport ATPase. Vice versa it indicates that this kinase is involved in the regulation of the Ca^{2+} transport ATPase activity. At 20 °C 1 mg of sarcoplasmic reticulum binds ca. 10 nmol of Ca^{2+} (Fiehn and Migala, 1971; Ikemoto, 1974, 1975). If 1 mg of vesicular protein contains ca. 10 μg of kinase (see Hörl and Heilmeyer, 1978) and if the membrane bound enzyme binds 12 mol of Ca^{2+}/mol of kinase as purified phosphorylase kinase (Kilimann and Heilmeyer, 1977), it can be estimated that maximally 0.2 nmol of Ca^{2+}/mg of vesicular protein is bound to the Ca^{2+}-dependent protein kinase. Although it represents a small amount of the total Ca^{2+} bound to sarcoplasmic reticulum, it may exert a regulatory function if this kinase is involved in the regulation of the Ca^{2+} transport ATPase.

The antibodies against phosphorylase kinase inhibit both the Ca^{2+}-dependent, membrane-associated, protein kinase as well as the Ca^{2+} transport ATPase. Even though these antibodies are heterogeneous—they contain antibodies against each of the three subunits α, β, and γ of phosphorylase kinase—it can be excluded that they cross-react with any of the main components, including the ATPase of the sarcoplasmic reticulum. In addition anti-γ does not cross-react with rabbit muscle actin. This latter protein was shown to be very similar if not identical with the γ subunit of dog fish phosphorylase kinase (Fischer et al., 1975). This specificity of the anti-phosphorylase kinase suggests that the inhibition of the membrane associated kinase causes the ATPase inhibition; i.e., the kinase activates the ATPase.

Anti-phosphorylase kinase does not influence the ratio of the Ca^{2+} uptake rate to the ATPase activity, whereas phosphorylase *b* reduces the Ca^{2+} uptake rate to a higher degree than the Ca^{2+} transport ATPase activity (compare Figures 3 and 5). Probably the Ca^{2+}-dependent protein kinase and protein phosphatase form an additional ATPase by turning over glycogen phosphorylase *b* and *a* which only produces an apparent uncoupling of the Ca^{2+} transport ATPase activity. Assuming that the membrane-associated, Ca^{2+}-dependent, protein kinase can be phosphorylated autocatalytically at the same rate as phosphorylase kinase (Wang et al., 1976), the presence of an excess of general protein phosphatase activity which is able to dephosphorylate the enzyme would form an additional Ca^{2+}-dependent ATPase. However, the low amount of the Ca^{2+}-dependent, membrane-bound kinase (10 μg/mg of SR, see above) in the vesicles would yield an ATPase activity of ca. 1×10^{-5} μmol min^{-1} (mg of SR)$^{-1}$, i.e., 0.001% of the Ca^{2+} transport ATPase activity. Both phosphorylase *b* and anti-phosphorylase kinase reduce the maximal Ca^{2+} accumulation, the latter agent to a higher degree than the former. The steady-state level of accumulated Ca^{2+} represents an equal rate of Ca^{2+} efflux and inward transport (Inesi, 1972). Therefore, a partially inhibited Ca^{2+} uptake rate must result in a decrease of the steady-state level (see Figure 5).

The parallel inhibition of phosphorylase kinase and of the Ca^{2+} transport ATPase by hydroxylamine (see Figure 8) indicates that the Ca^{2+} transport ATPase is regulated by the Ca^{2+}-dependent protein kinase. Removal of hydroxylamine completely reactivates the Ca^{2+} transport ATPase activity and allows phosphoprotein formation (see Figure 9 and Inesi et al. (1970)). Apparently in the native enzyme a hydroxamic acid derivative of the Ca^{2+} transport ATPase which would be expected to irreversibly destroy activity cannot be formed. However, this reaction is possible following trichloroacetic acid denaturation (Post and Kume, 1973). It is known that the Ca^{2+}-dependent protein kinase, phosphorylase kinase, incorporates phosphate into protein as phosphoserine or phosphothreonine. Hydroxylamine is not expected to react with these phosphoamino acids which could explain the observed reversibility of the hydroxylamine inhibition. Similarly, 0.2 M hydrazine reversibly inhibits to a lesser extent phosphorylase kinase activity (not shown). Therefore neither the Ca^{2+} uptake nor the ADP–ATP exchange reaction nor the ATPase activity are irreversibly destroyed by this latter amine (Makinose, 1969). An inhibition of the Ca^{2+}-dependent protein kinase activity can be correlated with a decrease of the Ca^{2+} transport ATPase activity. A similar but reversed relationship is expected to exist between the protein phosphatase and ATPase activity. In agreement with this hypothesis, the protein phosphatase inhibitors, oxalate and fluoride, cause an enhancement of the Ca^{2+} transport ATPase activity due to indirectly favoring the kinase reaction. Both inhibitors maximally stimulate the transport ATPase activity at a concentration which inhibits the protein phosphatase 70% to 80% (for fluoride inhibition, compare Hurd, 1967). The decrease of the intravesicular Ca^{2+} concentration by precipitation of calcium oxalate or calcium fluoride as discussed earlier may also synergistically stimulate the Ca^{2+} transport ATPase activity. If the intravesicular precipitation of calcium would be the only responsible effect for the ATPase activation fluoride should activate the ATPase at a lower concentration than oxalate since the solubility product of calcium fluoride is ca. two orders of magnitude lower than that of calcium oxalate. However, the influence of other components of the incubation mixture like Mg^{2+} or phosphates on the solubility product of CaF_2 is not known. In contrast a ca. fourfold higher concentration of fluoride (20 mM) in comparison with oxalate is needed to maximally stimulate the Ca^{2+} transport ATPase activity which could be better explained by the concentration dependent effects of these anions on the protein phosphatase (see Figures 6 and 7). A further increase of the concentration of both anions above a maximally stimulatory concentration leads to a decrease of the ATPase activity. This might be due either to a partial inhibition of the Ca^{2+}-dependent protein kinase (see Figures 6 and 7) or to a direct effect on the Ca^{2+} transport ATPase or to changes in the free Ca^{2+} concentration (even though the solubility product was exceeded with fluoride in the incubation mixture, no apparent precipitation of CaF_2 was observed; see above).

Hydroxylamine and oxalate act antagonistically on this ATPase probably mediated by the two regulatory enzymes, the kinase and the phosphatase. The ATPase activity should be changeable in a predictable way by the relative concentra-

tion of these two effectors. This prediction has been verified on vesicles which do not accumulate Ca^{2+} due to treatment of the vesicles at pH 7.5 (see Figures 6 and 10). Reduction of the Ca^{2+}-dependent protein kinase activity with hydroxylamine then leads to an inhibition of the Ca^{2+} transport ATPase activity which now can be reversed by an inhibitor of the protein phosphatase, oxalate (see Figure 10). Under these conditions a higher ratio of the Ca^{2+} to the oxalate stimulated ATPase activity of ca. 4 than that observed with intact vesicles of ca. 2 can be produced. It shows again that oxalate acts via the protein phosphatase on the Ca^{2+} transport ATPase which should only be possible when the Ca^{2+}-dependent protein kinase has phosphorylated the enzyme. In agreement with this interpretation at very low concentrations of ATP (0.1–1 μM) which probably are not sufficient to activate the Ca^{2+}-dependent protein kinase (K_M for ATP of phosphorylase = 400 μM; Jennissen and Heilmeyer 1974), no oxalate stimulation of the Ca^{2+} transport ATPase was observed (Yamada et al., 1971). These data indicate the participation of these two regulatory enzymes in the adjustment of the Ca^{2+} transport ATPase activity as observed indirectly with effectors. Effects of phosphorylase phosphatase and phosphorylase kinase will more directly show this interaction (Hörl and Heilmeyer, 1978).

Acknowledgments

We are indebted to Professor W. Hasselbach for many helpful suggestions during the course of this work and to Professor A. W. Holldorf for help in the preparation of the manuscript. The excellent technical help of Miss R. Wittal and Mrs. G. Botzet is gratefully acknowledged. Professor J. Schmidt-Thomé, Farbwerke Hoechst AG (Frankfurt), helped provide the sheep for immunization which is gratefully acknowledged.

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Evidence for the Participation of a Ca^{2+}-Dependent Protein Kinase and Protein Phosphatase in the Regulation of the Ca^{2+} Transport ATPase of the Sarcoplasmic Reticulum. 2. Effect of Phosphorylase Kinase and Phosphorylase Phosphatase†

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ABSTRACT: Dialysis of membranes of the sarcoplasmic reticulum against EDTA solubilizes polypeptides of molecular weight 140 000 and 120 000, corresponding to the α and β subunits of phosphorylase kinase; 100 000, phosphorylase *b* or ATPase; 58 000, calsequestrin; and 31 000, acidic proteins. Fourteen percent of the Ca^{2+}-dependent protein kinase and 13% of the protein phosphatase activity remain associated with these membranes. As a result the oxalate/Ca^{2+} activity ratio of the Ca^{2+} transport ATPase decreases from 1.9 to 1.1. Upon readdition of purified phosphorylase kinase and phosphorylase phosphatase this ratio increases to 1.8. Similarly, the oxalate/Ca^{2+} activity rate is also increased ca. twofold in untreated vesicles. Addition of phosphorylase phosphatase to vesicles isolated under conditions which optimally preserve the Ca^{2+}-dependent protein kinase and protein phosphatase activity reduces the Ca^{2+} transport ATPase activity maximally to 90%. Correspondingly the oxalate/Ca^{2+} activity ratio increases ca. ninefold. Phosphorylase phosphatase is also able to reduce the Ca^{2+} uptake rate by ca. 70% as measured in presence of 0.5 mM oxalate. The ATPase inhibition caused by phosphorylase phosphatase can be specifically reversed by phosphorylase kinase as a function of its concentration. The effect of phosphorylase is a function of the age of the vesicles. During 15 days the ATPase is desensitized and only minimally responds to phosphorylase phosphatase addition. The amount of incorporated phosphate can be reduced by antiphosphorylase kinase by 59% and by phosphorylase phosphatase by 39%. Phosphorylase kinase alone increases the degree of phosphorylation by 7%.

It has been shown that an inhibition of a Ca^{2+}-dependent protein kinase which is present in purified sarcoplasmic reticulum from rabbit skeletal muscle either with anti-phosphorylase kinase or with hydroxylamine leads to a concomitant inhibition of the Ca^{2+} transport ATPase (Hörl et al., 1975a, 1978; Hörl and Heilmeyer, 1976a,b; Gröschel-Stewart et al., 1976). The Ca^{2+} uptake rate and maximal Ca^{2+} accumulation are reduced parallel to a decrease of the Ca^{2+} transport ATPase activity caused by anti-phosphorylase kinase or phosphorylase *b* (Hörl et al., 1975b, 1978). Oxalate or fluoride stimulates the Ca^{2+} transport ATPase activity which can be correlated with an inhibition of the protein phosphatase. The antagonistic effects of hydroxylamine and oxalate on the ATPase activity suggest that the ATPase activity is regulated by the activity ratio of a Ca^{2+}-dependent protein kinase and protein phosphatase (Hörl et al., 1978).

A protein kinase which is activated by Ca^{2+} concentrations between 10^{-8} and 10^{-5} M and which accepts several proteins as substrate is phosphorylase kinase (Heilmeyer et al., 1970; Brostrom et al., 1971; Kilimann and Heilmeyer, 1977); a general protein phosphatase seems to be phosphorylase phosphatase (Antoniw and Cohen, 1975; Djovkar and Heilmeyer, 1978). Since the former enzyme is available as homogeneous protein and the latter as highly enriched preparation, it seemed

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0006-2960/78/0417-0766$01.00/0