Regulation of the Sarcoplasmic Reticular Ca^{2+} Transport ATPase by Phosphorylation and Dephosphorylation

Ludwig M. G. Heilmeyer, Jr. and Magdolna Varsanyi

Institut für Physiologische Chemie, Lehrstuhl I, Ruhr-Universität, Postfach 10 21 48, D-4630 Bochum 1

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At 0.1 mg/ml protein and 0.45 μM free Ca^{2+} 1 mol trichloroacetic acid precipitable phosphate is incorporated into 100,000 g SR protein as hydroxilamine sensitive acylphosphate. At nearly physiological protein concentration (ca. 7 mg/ml) a total of ca. 0.8 mol phosphate/100,000 g protein is incorporated, from which a fraction of 0.3 mol/100,000 g protein is insensitive to the hydroxylamine treatment, *i.e.* it is alkylphosphate. Phosphorylase kinase accelerates the alkylphosphate incorporation ca. 3-fold and enhances its final level to 0.7 mol/100,000 g protein. At 1.6 nM free Ca^{2+} alkylphosphate incorporation occurs at high SR concentration to a maximal extent of 0.5 mol/ 100,000 g protein. The incorporated alkylphosphate is present in comparable amounts in the 100,000 M_R Ca^{2+}-transport ATP-ase and a polypeptide of M_R 9,000.

Immunofluorescence localization studies first indicated that an antigen identical or related to the Ca^{2+}-calmodulin dependent protein kinase, phosphorylase kinase, is associated with rabbit skeletal muscle membranes [1 – 3]. When sarcoplasmic reticulum (SR) is isolated from these muscles phosphorylase kinase and additionally phosphoprotein phosphatase activity cannot be completely removed even by repeated sucrose gradient centrifugation [1]. The subunit structure of phosphorylase kinase is $(\alpha\beta\gamma\delta)_4$ (review [4]); its δ-subunit is equivalent to calmodulin [5] and represents probably the only Ca^{2+}-binding protein of this enzyme [6]. Polypeptides corresponding in molecular weight to those of the α and β subunits but not to that of the γ subunit could be demonstrated in SR by SDS gel electrophoresis [7]. In the rabbit system it cannot be decided if indeed a separate membrane bound Ca^{2+}-calmodulin dependent protein kinase exists since contamination of the SR membranes with soluble phosphorylase kinase cannot be excluded. However, SR vesicles isolated form I-strain mice muscles which lack cytoplasmic phosphorylase kinase contain the same amount of Ca^{2+} dependent and independent protein kinase activity as vesicles isolated from normal mice muscles [8]. These histological and biochemical localisation studies indicate that in addition to phosphorylase kinase SR membranes may contain other Ca^{2+}-calmodulin dependent and independent protein kinases.

A well characterized component of the rabbit skeletal muscle SR, calsequestrin, when isolated behaves like a Ca^{2+}-calmodulin stimulated protein kinase [9]. It can selfphosphorylate calsequestrin if endogeneous phosphate is removed by preincubation with a protein phosphatase; up to 0.5 mol phosphate are incorporated per mol protein [10].

Calsequestrin as well as phosphorylase kinase seem to belong to the class of peripheral membrane associated proteins as can be concluded from their behaviour in a protein glycogen complex [11, 12]. One major component of this complex represents glycogen particles *i.e.* glycogen metabolizing enzymes together with their respective interconverting enzymes adsorbed to the carbohydrate, the other SR membranes [11]. Both, phosphorylase kinase and calsequestrin, are found to be associated with the glycogen particles as well as with the SR membranes [12, 13]. For phosphorylase kinase its presence in either of these two compartments seems to be a function of the free Ca^{2+} concentration [14]. At μM free Ca^{2+} the enzyme associates with SR whereas it dissociates from these membranes at nM free Ca^{2+} [14]. The complementary enzyme, phosphoprotein phosphatase, behaves analogously: it also associates with and dissociates from these membranes at μM and nM free Ca^{2+}, respectively [15].

These observations lead to the idea that the Ca^{2+}-calmodulin dependent protein kinases e.g. phosphorylase kinase or calsequestrin as well as

Requests for reprints to Prof. Dr. Ludwig M. G. Heilmeyer, Jr.

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phosphoprotein phosphatase are functionally operative in both organelles: the SR membranes and the glycogen particles. If it is correct one could expect that these interconverting enzymes influence SR function *i.e.* Ca^{2+} transport across these membranes.

Ca^{2+} uptake into SR vesicles is catalyzed by the Ca^{2+} transport ATPase (review [16]). A close relationship between this ATPase and the interconverting enzymes became apparent from the following observation: Antibodies directed against phosphorylase kinase inhibit the Ca^{2+} transport ATPase activity even though they do not directly interact with this enzyme [17]. It was concluded that the activity of the Ca^{2+} transport ATPase is a function of the balance between protein kinase and protein phosphatase activities. An alternate experiment points into the same direction: protein phosphatase addition to SR vesicles can reduce the Ca^{2+} transport ATPase activity maximally ca. 90%. This inhibition can be overcome by subsequent addition of the counteracting enzyme, phosphorylase kinase; the enzyme can be reactivated to more than 50% [17]. An explanation could be that the protein kinase phosphorylates and thereby activates the Ca^{2+} transport ATPase and vice versa that the protein phosphatase reverses both processes. Further indirect proof for this hypothesis was obtained from competition experiments. It would be expected that the phosphorylated Ca^{2+} transport ATPase competes with an exogeneously added phosphorylated protein on the protein phosphatase. Indeed, at high SR concentration in presence of μM free Ca^{2+} and ATP/Mg^{2+} *i.e.* under conditions at which the Ca^{2+} transport ATPase can be phosphorylated and the protein phosphatase associates with SR (see above) an added soluble ^{32}P labelled protein is not dephosphorylated. Liberation of radioactivity *i.e.* dephosphorylation starts immediately when Ca^{2+}-calmodulin dependent phosphorylation reactions are blocked by sequestring the added Ca^{2+} with EGTA and concommitant dissociation of the protein phosphatase from the membranes can occur. The kinetic analysis of this phenomenon reveals a competitive protein phosphatase inhibition which is only observed above 5 mg SR/ml and which is maximally expressed between 7 and 10 mg/ml [15]. All these observations strongly suggest that the Ca^{2+} transport ATPase can be phosphorylated and dephosphorylated. The specificity of the protein kinase and protein phospatase employed in these studies would demand that the Ca^{2+} transport ATPase becomes phosphorylated at a serine or threonine residue, *i.e.* that an intermediate phosphoester linkage is formed.

Phosphorylation of the Ca^{2+} transport ATPase is well known to occur at an aspartyl residue; it is believed to be a catalytic intermediate of the enzymatic cycle (review [18]). If phosphorylation of SR is carried out at low protein concentration (0.1 mg/ml) the total amount of incorporated phosphate can be released by incubation with hydroxylamine, *i.e.* it behaves like acylphosphate [19]. A low amount of phosphate (ca. 0.02 mol/100,000 g protein) remains bound to the protein [20]. Such a low amount of esterphosphate was observed earlier and shown to be present on several polypeptides [21 – 23]. They might represent phosphorylatable enzymes, known contaminants of SR preparations. A high amount of hydroxylamine stable phosphate *i.e.* alkylphosphate is incorporated into membrane proteins if the same experiment as described above is carried out at high protein concentration (7 mg/ml) [20]. In presence of μM free Ca^{2+} and ATP/Mg^{2+} the amount of incorporated alkylphosphate increases rapidly during 3 min and reaches a final level of 0.3 mol/100,000 g protein; at nM free Ca^{2+} this final level is even higher (0.5 mol/(100,000 g protein). Only at μM free Ca^{2+} phosphorylase kinase enhances the initial rate as well as the final amount of incorporated alkylphosphate 2 to 3 fold.

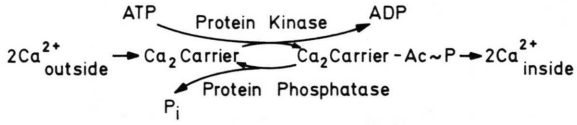
Alkylphosphate is incorporated into two polypeptides: one represents the 100,000 M_r Ca^{2+} transport ATPase, the other a 9,000 M_r polypeptide. Phosphorylase kinase enhances phosphate incorporation into the Ca^{2+} transport ATPase exclusively, it cannot catalyze a further phosphorylation of the 9,000 M_r ploypeptide.

Purified Ca^{2+} transport ATPase can be phosphorylated by phosphorylase kinase as well; the degree varies somewhat with the preparations of both the ATPase and the kinase. As an average between 0.6 and 0.9 mol alkylphosphate are incorporated per mol ATPase. Without added phosphorylase kinase the purified ATPase is phosphorylated at a slow rate.

Only minute amounts of additional ester phosphate can be incorporated into SR proteins by the catalytic subunit of the cAMP dependent protein kinase at nM free Ca^{2+}; however, very surprisingly it enhances the low level of acylphosphate (< 0.1 mol/100,000 g protein) to ca. 0.7 mol/100,000 g protein.

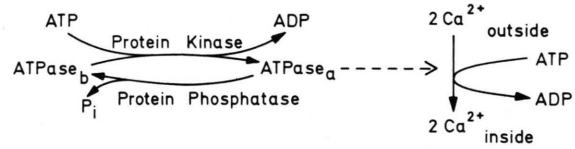
Based on the known specificity of this kinase again phosphate incorporation in form of serine or threonine ester would be excepted. Assuming that the cAMP dependent protein kinase cannot directly phosphorylate the aspartyl residue – it would be a very unusual reaction for this kinase – an alkylphosphate should be the primary reaction product. One might think that the phosphate groups must hen be transferred subsequently to the aspartyl residue.

To summarize: Protein kinases seem to be involved in several steps related to regulation and transport of Ca^{2+} across SR membranes. The catalytic subunit of the cAMP dependent protein kinase seems to be able to stimulate directly or indirectly the formation of acylphosphate. To account for this observation it could be assumed that the ATPase is composed of three components: a protein kinase, a Ca^{2+} carrier and a protein phosphatase. They might be combined in the following reaction sequence:



Even though this scheme might probably oversimplified, it has the advantage to introduce the possibility that a protein kinase and protein phosphatase might be directly involved in the catalytic cycle of the Ca^{2+} transport ATPase.

Regulation of this ATPase activity could occur by additional phosphorylation at serine or threonine residues in an analogous fashion to that occurring during interconvèrsion of glycogen phosphorylase b and a:



Only the phosphorylated *a* form would be active and is then able to transport Ca^{2+} across the membranes with concomitant hydrolysis of ATP. Again it might be an oversimplified model, however, it could serve as a hypothesis for future studies in the search for a function of incorporated alkylphosphate into the Ca^{2+} transport ATPase.

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