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Modulation by phosphorylation of glycogen phosphorylase-sarcoplasmic reticulum interaction

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Glycogen phosphorylase *b* at concentrations close to those found in skeletal muscle interacts with sarcoplasmic reticulum membranes, but not with liposomes made of lipids extracted from these membranes, and is inhibited upon binding to the membrane. The interaction of glycogen phosphorylase with the sarcoplasmic reticulum membrane is modulated by phosphorylation, for the *a* form of this enzyme shows a $K_{0.5}$ of interaction about 10-fold lower than the *b* form. Upon association to the membrane the fluorescence properties of the coenzyme of glycogen phosphorylase, pyridoxal-5'-phosphate, are strongly altered, for the fluorescence at 535 nm is partially quenched and the fluorescence at 415–420 nm increases. Using fluorescein labeled sarcoplasmic reticulum membranes we have found that the average conformation of the $Ca^{2+}+Mg^{2+}$-ATPase is also altered on binding of phosphorylase *b*. In conclusion, the results reported in this paper suggest that glycogen phosphorylase and $Ca^{2+}+Mg^{2+}$-ATPase directly interact under experimental conditions similar to those found in the sarcoplasm, and that this interaction is modulated by phosphorylation of the phosphorylase.

Glycogen phosphorylase; Sarcoplasmic reticulum; $Ca^{2+}+Mg^{2+}$-ATPase; Fluorescein; Regulation

1. INTRODUCTION

In skeletal muscle glycogen is associated with relevant enzymes involved in its metabolism [1–3]. These glycogen particles also associate with the sarcoplasmic reticulum (SR)[1] membranes [3,4]. The major determinants of this association remain unclear, but there are functional gains. For example, stimulation of glycogen phosphorylase *b* kinase is faster when associated to the SR than in the soluble enzyme [4].

The integrity of glycogen plays an important role in maintaining an effective association of the glycogen particle with SR vesicular preparations [3], but glycogen phosphorylase and phosphorylase *b* kinase also remain tightly bound to preparations of the SR membrane, even when these are largely devoid of glycogen [3–5]. A large fraction (amounting to about 30–40%) of glycogen phosphorylase is likely to be tightly bound in vivo to the SR membrane and SR membrane preparations are largely contaminated by glycogen phosphorylase [3,6].

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Abbreviations: ATPase, $Ca^{2+}+Mg^{2+}$-ATPase; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FITC, fluorescein isothiocyanate; IU, μmols product per min per mg protein; PLP, pyridoxal-5'-phosphate; SR, sarcoplasmic reticulum; Tes, 2-[(2-hydroxy-1,1-bis-(hydroxymethyl) ethylamine] ethanosulfonic acid; Tris, tris(hydroxymethyl)-aminomethane

In this communication we report experimental observations which show that glycogen phosphorylase interacts with the SR at pH and ionic strength similar to those found in the sarcoplasm, and that this interaction is modulated by the phosphorylation state of phosphorylase and inhibits the activity of phosphorylase *b*.

2. MATERIALS AND METHODS

SR vesicles and purified glycogen phosphorylase *b* have been prepared as described elsewhere [8,9]. Glycogen phosphorylase *b* was found to be more than 98% pure on the basis of SDS gel electrophoretic patterns. Because SR preparations usually contain a significant contamination of glycogen phosphorylase we have carefully tested that the preparations used in this study were devoid of the 97500 Da band in SDS gel electrophoresis. The protein concentration was determined following the method of Lowry using bovine serum albumin as a standard [10], and also spectrophotometrically using an extinction coefficient at 280 nm for glycogen phosphorylase of $E^{1\%}_{1cm}$ = 13.2 at pH 6.9 [11]. Lipids were extracted from SR membranes by repeated washing with $HCCl_3/HOCH_3/H_2O$ 2/ 1/1 v/v. The functional state of SR vesicles has been characterized as in [12]. Only preparations of SR showing, at least, 4-fold stimulation of the ATPase activity by calcimycin (1 μg/ml) at 22°C were used in this study, e.g. only well sealed SR vesicles. On average, the $Ca^{2+}+Mg^{2+}$-ATPase activity of leaky SR vesicles was 3–4 μmols ATP hydrolyzed per min per mg protein at 22°C. Phosphorylase *a* was prepared in a reaction mixture containing 8 $mg \cdot ml^{-1}$ phosphorylase *b*, 70 $\mu g \cdot ml^{-1}$ phosphorylase kinase, 18 mM disodium glycerol-2-phosphate, 18 mM 2-mercaptoethanol, 1 mM ATP, 10 mM magnesium acetate (pH 6.8). The reaction was run until the phosphorylase activity ratio without AMP, caffeine plus AMP remained constant at about (≥0.90). The reaction was stopped by an equal volume of 10 mM EDTA, 1 mM EGTA, 5 mM disodium glycerol-2-phosphate, 5 mM 2-mercaptoethanol (pH 6.8) [13].

Glycogen phosphorylase was assayed in the direction of glycogen synthesis in the presence of 10 mM glucose-1-phosphate, 0.45 g·l^{-1} glycogen, 0.1 mM AMP, 1 mM EGTA, 0.15 M KCl, 10 mM TES (pH 7.4), and phosphorylase (0.1 mg·ml^{-1}). Inorganic phosphate was determined according to the method of Fiske and SubbaRow [14]. Phosphorylase activity in the direction of glycogen breakdown was measured as in [15], using the following reaction mixture: 12 mM H_2PO_4K, 10 mM magnesium acetate, 0.63 mM $NADP^+$, 50 mM imidazole, 1 mM AMP, 0.45 g·l^{-1} glycogen, 1 IU phosphoglucomutase, 1 IU glucose-6-phosphate dehydrogenase (pH 6.9). Due to problems of rapid $NADP^+$ consumption, in the presence of high concentrations of glycogen phosphorylase we have measured its activity using the light scattering method indicated in [9], the light scattering being monitored by turbidity measurements under the experimental conditions indicated above. The experimental approach followed was as outlined in [9], except that glycogen itself was used as primer and the reaction has been initiated by addition of AMP. Because we found that an addition of 1 mM AMP produces a relatively fast reaction, i.e. the light scattering change stopped in about 3–4 min, we have used a lower concentration of AMP in these assays, in order to allow for a much better accuracy in our estimations of the initial rate of light scattering change. The rest of the concentrations of relevant kinetic species in assay medium was identical to those used in Gutierrez-Merino et al [9].

The labeling of the SR vesicles with FITC and the determination of the extent of labeling were carried out as indicated elsewhere [8,16].

Fluorescence measurements were carried out using a spectrofluorimeter Hitachi-Perkin Elmer, model 650-40, equipped with a thermostated cell holder.

2.1. *Chemicals*

Bovine serum albumin, AMP, ATP, phosphoenolpyruvate, EGTA, FITC, phenylmethyl sulfonyl fluoride, β-mercaptoethanol, sephadex G-50, sodium dodecyl sulfate, glycogen, glucose 1-phosphate, NADH, $NADP^+$, Tris (TRIZMA base), and TES were obtained from Sigma. Calcimycin, pyruvate kinase, lactate dehydrogenase, phosphoglucomutase and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim. All the other chemicals used in this study were obtained from Merck.

3. RESULTS AND DISCUSSION

We have studied the effect of SR on the activity of glycogen phosphorylase. Relatively high concentrations of glycogen phosphorylase are required in order to form the complex with SR membranes and under these conditions the spectrophotometric assay of the activity of this enzyme becomes unsuitable, for $NADP^+$ is rapidly exhausted. Therefore, we have directly monitored the release of phosphate assaying the enzyme in the direction of glycogen synthesis (see Section 2). As shown in Fig. 1, when phosphorylase *b* is used the synthesis of glycogen is decreased by the presence of SR membranes in the reaction mixture. This regulatory effect of SR membranes is much lower when measured with the *a* form. From these results we have estimated a value of apparent $K_{0.5}$ of association between glycogen phosphorylase *b* and SR membranes of 0.8–1.0 mg/ml in these experimental conditions. The apparent $K_{0.5}$ of the *a* form is about 10-fold lower. Using ultracentrifugation (60 min at 35 000 × *g*) to separate membrane-bound from free enzyme we have obtained direct evidence that under these experimental conditions at 1 mg phosphorylase *b*·ml^{-1} and 1 mg membrane protein·ml^{-1} only about 40% of total phosphorylase *b* is bound to the SR membrane, in good agreement with the extent of inhibition produced under these experimental conditions. From the results presented in Fig. 1, a maximum inhibition of ca. 80–90% of the glycogen phosphorylase *b* activity is attained in the presence of 4 mg SR protein·ml^{-1}. This result is in good agreement with earlier findings [4,6]. Fig. 1 also shows that neither liposomes made of lipids extracted from the SR membrane, nor liposomes made of egg lecithin, significantly inhibit the phosphorylase in identical experimental conditions. Therefore, it follows from these results that the regulatory effect of the SR membrane is specific and not due to interaction of the phosphorylase *b* with the lipid bilayer.

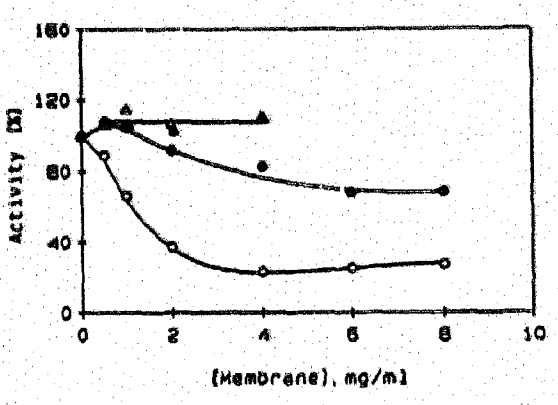

Fig. 1. Dependence of the activity of glycogen phosphorylase upon the concentration of SR membranes (○,●) and of liposomes of SR lipids (△) and of egg lecithin (▲). The concentration of SR membranes is given in mg protein·ml^{-1} and that of lipids in mg lipid·ml^{-1}. On average the SR membrane contained 0.6 mg lipid per mg protein. Experimental conditions: 10 mM TES (pH 7.4)/0.15 M KCl/10 mM glucose-1-phosphate/ 0.45 g·l^{-1} glycogen/ 1 mM EGTA/ 0.1 mM AMP, and 0.1 mg·ml^{-1} of phosphorylase *a* (●) or *b* (○). Temperature 25°C.

The results shown in Fig. 2 are consistent with the conclusions indicated above, and in addition they show that the limiting size of glycogen particles (monitored by the limiting scattering change) is decreased by the presence of SR membranes. The $K_{0.5}$ of this effect is only slightly dependent upon the temperature from 25 to 37°C. On the other hand, this parameter is not largely altered when measured with the *a* form in the presence of up to 1 mg SR protein·ml^{-1}, in good agreement with the results shown in Fig. 1. Furthermore, we have found no effect of erythrocyte membranes (up to 2 mg protein·ml^{-1}) on this parameter. On the basis of the structure of the SR membrane these results suggest a likely interaction between phosphorylase *b* and the Ca^{2+} + Mg^{2+}-ATPase, for this is the only protein of this membrane that is outwardly oriented [17]. Therefore, the possibility that this interaction alters the conformation of both enzymatic systems has been explored.

Upon mixing glycogen phosphorylase *b* with purified SR membranes the emission of fluorescence of PLP is

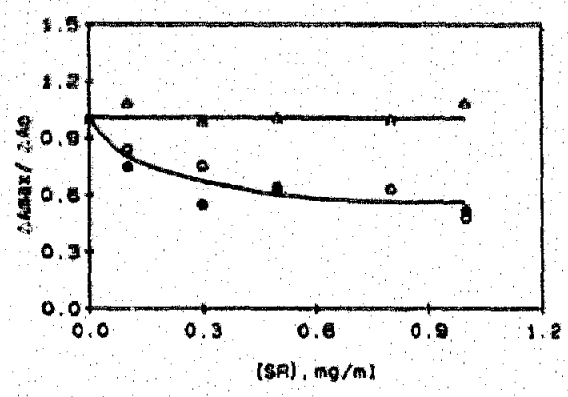

Fig. 2. Dependence upon the concentration of SR membranes of the turbidity change at 400 nm associated to glycogen synthesis catalysed by phosphorylase *b* (○,●) and *a* (△). The maximum turbidity change (A_{max}) has been normalized with respect to the value (A_0) measured in the absence of SR membranes. The assay medium contained: 10 mM TES (pH 7.4), 2.5 mM glycogen (in glucose units), 10 mM glucose-1-phosphate, 0.1 mM AMP and phosphorylase *b* or *a* 1 mg·ml^{-1}. Temperature 25 (○,△) and 37°C (●).

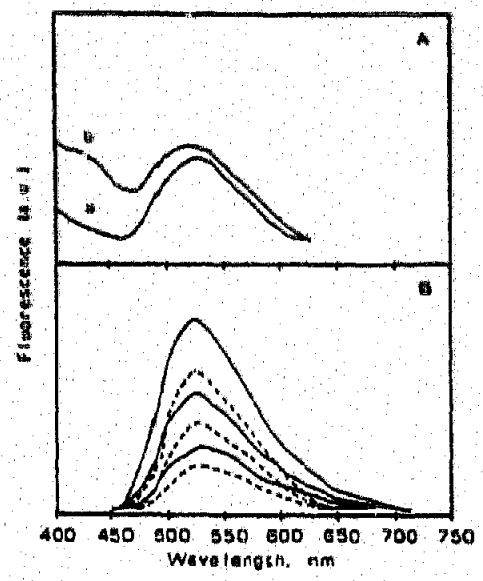

Fig. 3. Panel A: Fluorescence emission spectra (excitation wavelength, 335 nm) of glycogen phosphorylase *b* in buffer (a), and in the presence of SR membranes (b); a.u. stands for arbitrary units. Experimental conditions: glycogen phosphorylase *b* concentration (0.1 mg/ml) (a,b), and SR membranes concentration (0.1 mg protein/ml) (b) in buffer: 10 mM Tris-acetate (pH 7.0)/50 mM β-mercaptoethanol. Temperature: 25°C. Spectrum (b) was recorded at approximately 10 min after addition of SR membranes to glycogen phosphorylase *b* in buffer. Panel B: Emission spectra (excitation wavelength 425 nm) of glycogen phosphorylase *b* in the absence (continuous line) and in the presence of SR membranes (0.8 mg protein·ml^{-1}) (dotted line). Concentration of glycogen phosphorylase *b* from top to bottom: 2.5, 1.5 and 0.8 mg·ml^{-1}. Other experimental conditions: 10 mM TES (pH 7.4), 25°C. The scattering signal from SR membranes was loaded into a microcomputer and subtracted from the overall fluorescence signal obtained to yield the spectra shown by dotted lines.

partially quenched, see Fig. 3. The spectral changes are readily evident from 1–2 min after mixing. It can be observed that the fluorescence at 535 nm, a fluorescence characteristic of PLP bound to the active enzyme [18], is about 40% quenched (Panel B), and that a band centered at about 415–420 nm is largely increased (Panel A). This effect has also been observed in the presence of allosteric ligands of glycogen phosphorylase *b* in the medium, such as AMP (50 μM), Mg^{2+} (10 mM), glucose (10 mM) or phosphate (10 mM) (results not shown).

To check a putative effect of glycogen phosphorylase on the conformation of the SR $Ca^{2+}+Mg^{2+}$-ATPase we have labeled SR membranes with FITC, a reagent that has been shown to specifically label the catalytic center of this ATPase under certain experimental conditions [19,20]. Covalently bound fluorescein has been shown to monitor the E1/E2 equilibrium distribution of the ATPase [21]. In particular, the E2 conformation shows an intensity of the fluorescence of fluorescein bound to the catalytic center about 10–12% higher than the E1 conformation [21]. The results obtained in the titration of labeled SR membranes with glycogen phosphorylase *b* are shown in Fig. 4. These results suggest that the conformation of the ATPase is altered upon binding of glycogen phosphorylase to the SR membrane. The possibility that the fluorescence changes observed could be due to fluorescence energy transfer to PLP can be discarded, for PLP emits at longer wavelengths, and in this case fluorescein should be the donor [22]. Therefore, it appears that glycogen phosphorylase *b* shifts the conformational equilibrium of the ATPase towards an E2-like conformational state, which is the conformation showing the highest intensity of fluorescence of fluorescein.

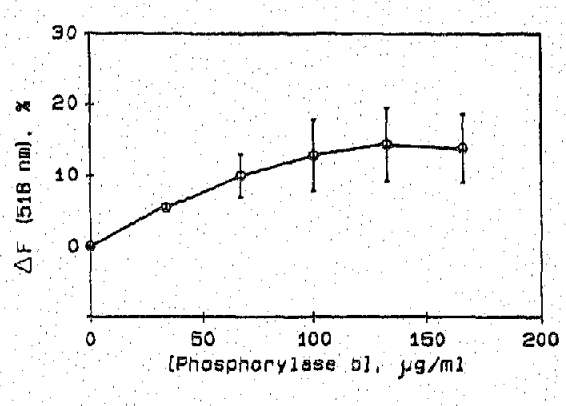

Fig. 4. Dependence upon the concentration of glycogen phosphorylase *b* of the fluorescence of SR membranes labeled with FITC (emission wavelength: 518 nm; excitation wavelength: 475 nm). Experimental conditions: SR (23 ± 2 μg protein/ml) in buffer 50 mM TES (pH 7.45), 0.1 M KCl, 0.25 M sucrose and 2 mM β-mercaptoethanol. Temperature: 20°C. The bars indicate the average fluctuations of the fluorescence change estimated from triplicate experimental series.

Glycogen phosphorylase *b* has been suggested to have hydrophobic binding domains, on the basis of bile salt binding and of stimulation of its activity by these compounds and by organic solvents [23,24] and Centeno, F., Fernandez-Salguero, P. and Gutierrez-Merino, C., (unpublished results), and the $Ca^{2+} + Mg^{2+}$-ATPase has been shown to be stimulated to about the same level (i.e. about 30–40%) by a variety of hydrophobic compounds [25–27]. Therefore, it is likely that glycogen phosphorylase *b* is effectively interacting with this hydrophobic binding region of the ATPase at concentrations close to those found in the sarcoplasm. The negligible effect of a temperature change from 25°C to 37°C upon the $K_{0.5}$ of interaction between phosphorylase *b* and SR membranes clearly supports this hypothesis.

In conclusion, this report shows that 2 of the most relevant protein components of the SR membrane - glycogenolytic complex (glycogen phosphorylase and $Ca^{2+} + Mg^{2+}$-ATPase), interact under physicochemical conditions that do not largely deviate from physiological conditions. In addition, this interaction appears to have clear functional relevance, because it induces an inactive form of the glycogen phosphorylase *b*, not strongly activatable by AMP, and is controlled by phosphorylation of this enzyme. It is suggested that this association can in vivo co-operate to potentiate muscle relaxation.

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