REGULATION OF PHOSPHOPROTEIN PHOSPHATASE BY PHOSPHORYLATION OF OTHER PROTEINS IN SKELETAL MUSCLE

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1. Introduction

The experiments with a protein–glycogen complex isolated from rabbit skeletal muscle showed that the activation of phosphorylase takes place synchronously with the inhibition of phosphoprotein phosphatase (phosphatase, EC 3.1.3.17) during the phosphorylation processes (flash activation) [1,2]. No clear explanation has been offered for the transient inhibition of phosphatase. Recently it become known that phosphatase activity can be inhibited by different proteins, e.g., nonactivated phosphorylase kinase [3], heat-stable inhibitors [4] and the regulatory subunit of cAMP-dependent protein kinase [5]. The phosphorylation of phosphorylase kinase [6] and one fraction of the heat-stable inhibitors [7,8] enhances their inhibitory efficiency.

In the present paper it is demonstrated that flash activation takes place in muscle extract, too. We suggest that transient inhibition of phosphatase is caused by the interaction of phosphatase with other proteins phosphorylated by cAMP-dependent protein kinase.

2. Methods

Rabbit skeletal muscle phosphorylase *a* was prepared and measured as in [9]. Phosphorylated glycogen synthase *b* was prepared and assayed as in [10]. The inhibitor protein of cAMP-dependent protein kinase was prepared by the method in [11]. Phosphorylase kinase activity was measured by the method in [12]. Protein was determined by the modified Lowry procedure [13].

Freshly excised rabbit skeletal muscle was homogenized in a Waring Blendor with 1.5 vol. 0.3 M sucrose, pH 7.4. After centrifugation at 6000 × *g* 10 ml supernatant was passed through a Sephadex G-25 column (1.5 × 50 cm) equilibrated and eluted with 0.3 M sucrose, pH 7.4. The fractions having the highest protein concentration were collected. All operations were carried out at 4°C.

The phosphorylation of proteins (flash activation) was studied at 30°C in an incubation medium containing: Sephadex G-25 filtrate, 5×10^{-6} M cAMP, 3×10^{-3} M Mg^{2+} and 9×10^{-4} M ATP or [γ-^{32}P]ATP, in the presence or absence of 5×10^{-3} M caffeine (final vol. 1.2 ml, protein 11 mg/ml, pH 7.4). Aliquots were withdrawn, stopped by the addition of 0.1 M NaF/0.04 M glycerophosphate/0.002 M EDTA, pH 6.8 and assayed for phosphorylase *a*, glycogen synthase *a* and phosphorylase kinase activity. Radioactivity incorporated in the proteins from [γ-^{32}P]ATP was measured as in [6]. Samples of the activation mixture (0.15 ml) were added to 0.05 ml phosphorylase *a* (0.4 mg) or glycogen synthase *b* (0.1 mg) in the presence of 5×10^{-3} M caffeine at 30°C and the phosphatase activity was assayed with both substrates [5]. Phosphatase activity was also checked in a sample diluted 20-fold with 0.3 M sucrose prior to addition to phosphorylase *a* or glycogen synthase *b*.

3. Results and discussion

As shown in fig.1 the addition of cAMP and Mg-ATP triggers an immediate formation of phospho-

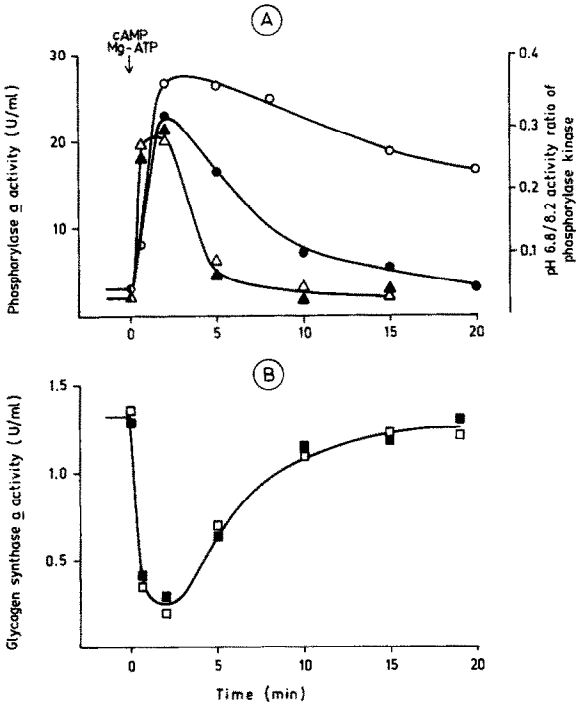

Fig.1. Activation of phosphorylase kinase, phosphorylase (A) and glycogen synthase (B) in a Sephadex G-25 filtrate. Conditions of activation process are described in section 2. Activity of phosphorylase kinase (△,▲), phosphorylase *a* (○,●), and glycogen synthase (□,■). Open symbols indicate the absence and closed symbols the presence of 5×10^{-3} M caffeine.

proteins, i.e., activated phosphorylase kinase, phosphorylase *a* and glycogen synthase *b*, simultaneously, and after 2–5 min the dephosphorylation reactions also take place. Caffeine accelerates the dephosphorylation of phosphorylase *a* but does not influence the dephosphorylation of other phosphoproteins. It seems that caffeine can eliminate the nucleotide (AMP, IMP) inhibition of the dephosphorylation of phosphorylase *a* [9].

During the activation process phosphatase activity was also tested using phosphorylase *a* and glycogen synthase *b* as substrates (fig.2). After a rapid, reversible inhibition the phosphatase activity begins to increase and after 8 min resumes its initial value in the undiluted samples. However, the phosphatase activity remained unchanged in 20-fold diluted samples indicating that the inhibition results from the interaction of phosphatase with some of the other components of the filtrate.

When the activation process was carried out in the presence of EGTA the inhibition of phosphatase was also observed, though the formation of phosphorylase *a* did not occur (not documented). This suggests that the inhibition is directly related to the phosphorylation of proteins catalyzed by protein kinase. It is known that cAMP-dependent protein kinase has an inhibitor protein which cancels its enzymic activity [11].

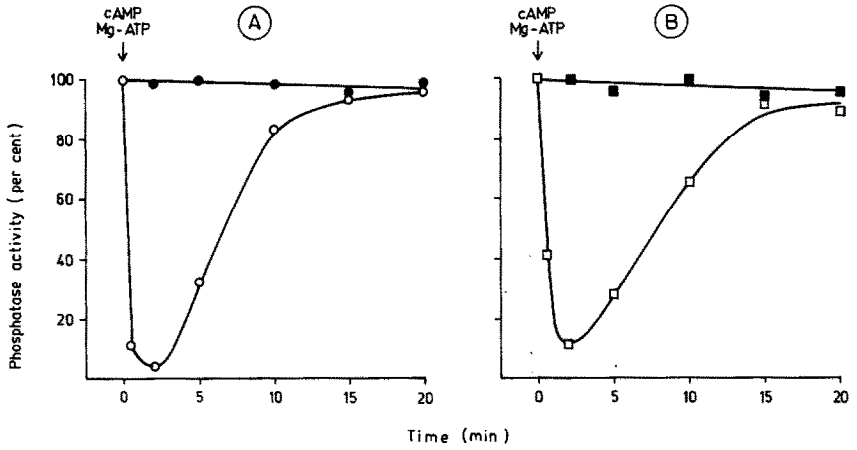

Fig.2. Reversible inhibition of phosphatase in a Sephadex G-25 filtrate. Phosphatase activities were tested for phosphorylase *a* (A) and glycogen synthase *b* (B). Conditions of the activation process and the phosphatase assay are described in section 2. Phosphatase activity is expressed as % initial activity obtained before addition of ATP. Phosphatase activity of undiluted (○,□) and 20-fold diluted (●,■) samples.

As seen in fig.3 in the absence of the inhibitor protein the radioactivity incorporated into proteins from [γ-^{32}P]ATP was maximum at 30 s, thereafter it slowly decreased. Synchronously the phosphatase activity rapidly decreased but later resumed its initial value. When the inhibitor protein was added to the reaction mixture, the incorporated radioactivity decreased to a very low level and the phosphatase activity remained unchanged. These results offer evidence for the control of phosphatase activity by the phosphorylation of proteins with cAMP-dependent protein kinase.

We have found two levels in the regulation of phosphatase. At the first level the control is exercised by phosphorylated phosphorylase kinase, phosphorylated heat-stable inhibitor(s) and, perhaps, the regulatory subunit of protein kinase. The second control comes into play at a later phase of phosphatase regulation. This control is by ligands (AMP, IMP, glucose, glucose 6-phosphate) and appears to affect only the dephosphorylation of phosphorylase *a* but not the dephosphorylation of other phosphoproteins. It is also of interest that the transient formation of different phosphoproteins and the reversible inhibition of phosphatase can also be observed in the muscle extract.

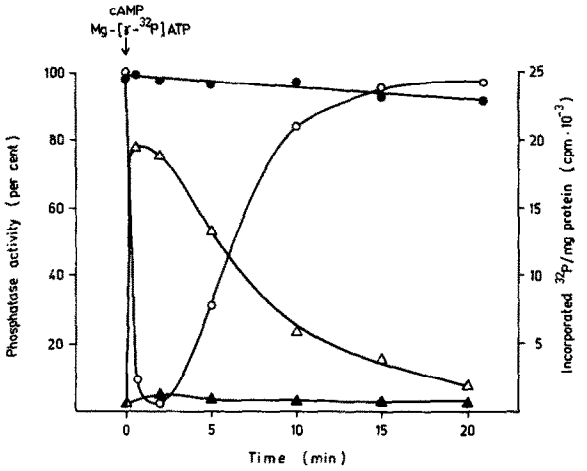

Fig.3. Effect of the inhibitor protein of cAMP-dependent protein kinase on the reversible inhibition of phosphatase. Experimental conditions are given in section 2. Radioactivity incorporated into protein (△,▲), activity of phosphatase (○,●) tested with phosphorylase *a*. Open symbols represent the absence and closed symbols the presence of 0.72 mg/ml inhibitor protein of cAMP-dependent protein kinase.

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