PHOSPHORYLATION OF GUINEA PIG CARDIAC NATURAL ACTOMYOSIN AND ITS EFFECT ON ATPase ACTIVITY

Y.S. Reddy and Leigh E. Wyborny

Cardiovascular Surgical Research Laboratories,
Texas Heart Institute
of St. Luke's Episcopal and Texas Children's Hospitals,
Texas Medical Center, and the Veterans Administration Hospitals,
Houston, Texas 77025

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SUMMARY: Guinea pig cardiac natural actomyosin incubated with commercial protein kinase, Mg^{2+}-ATP, and cyclic AMP produced little or no change in actomyosin ATPase activity. However, addition of sodium fluoride, a known phosphatase inhibitor resulted in a decreased actomyosin ATPase at all measured calcium concentrations. The presence of phosphatase activity in actomyosin and protein kinase was confirmed with p-nitrophenyl phosphate. These results indicate the importance of inhibiting phosphatase activity, particularly when measuring biological or enzymatic activity as a function of phosphorylation.

INTRODUCTION

The positive inotropic effects of catecholamines are mediated by cyclic AMP (1,2). Since cyclic AMP activates several protein kinases (3), it could be responsible for the effects of catecholamines on the contractile activity of the heart (4). It is now well established that the contractile proteins, myosin, troponin-I and troponin-T are capable of undergoing phosphorylation and dephosphorylation reactions (5). Cardiac troponin can be phosphorylated by either exogenous or endogenous cyclic AMP dependent protein kinase (6-8). The physiological role of phosphorylated proteins in the control of muscle contraction is as yet unclear. This study concerns the role of phosphorylation in the control of cardiac natural actomyosin ATPase activity.

MATERIALS AND METHODS: Cardiac natural actomyosin was prepared as described by Honig (9). Guinea pigs were killed by a blow on the head, the hearts were removed and immediately immersed in ice cold deionized water. The hearts were cut into small pieces and thoroughly rinsed to remove hemoglobin. The isolation procedures were carried out in a cold laboratory between 4-6° C in the presence of 0.5 mM dithiothreitol. The tissue was homogenized in six volumes of nitrogen equilibrated Weber-Edsall solution containing 10^{-6} M pepstatin, a catepsin-D inhibitor. After centrifugation for 30 minutes at 10,000 x g, natural actomyosin was precipitated from the

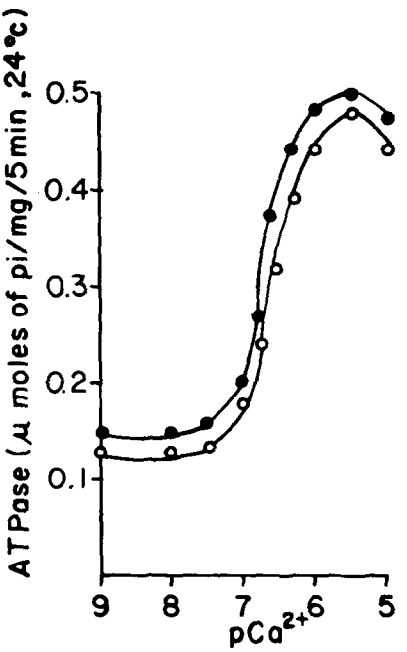

Figure 1. Effect of protein kinase and cyclic AMP on actomyosin in ATPase activity. Control actomyosin (0-0). Actomyosin pretreated with protein kinase (40-60 μg), Mg-ATP, and 10^{-5} M cyclic AMP for 120 minutes (●-●). Reaction medium for the actomyosin ATPase assay is the same as that described under Methods.

supernatant by dilution with ten volumes of cold glass distilled water. The precipitate was dissolved in Weber-Edsall solution, reprecipitated by dilution with ten volumes of cold deionized water, redissolved in Weber-Edsall solution, and reprecipitated by dilution. The final precipitate, which is known as purified natural actomyosin, was dissolved in a reaction medium, consisting of 100 mM Tris-HCl, 20 mM sodium azide, and 10mM $MgCl_2$, pH 7.6.

Natural actomyosin (20 mg) was incubated at room temperature for 120 minutes with 1.0 mg of commercial protein kinase (Sigma), 10^{-5} M cyclic AMP, 1.0 mM ATP, 30 mM sodium fluoride, 25 mM Tris-HCl, 5.0 mM sodium azide, 2.5 mM $MgCl_2$, pH 7.6. Following incubation, the natural actomyosin was collected by centrifugation at 2000 x g and then suspended in the reaction medium.

Dephosphorylated natural actomyosin was prepared with 9.0 mg of phosphorylated actomyosin incubated at room temperature with either 1.0 mg of protein kinase, 10^{-5} M cyclic AMP, 25 mM Tris-HCl, 2.5 mM $MgCl_2$, 5.0 mM sodium azide, pH 7.6 or 0.36 mg of alkaline phosphatase (1:15 enzyme to substrate ratio) in the presence of 400 mM KCl, 20 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 8.0. The actomyosin suspension was diluted with ten volumes of cold deionized water and collected by centrifugation at 2000 x g for 10 minutes. The resulting dephosphorylated natural actomyosin was collected, washed, and resuspended as described above.

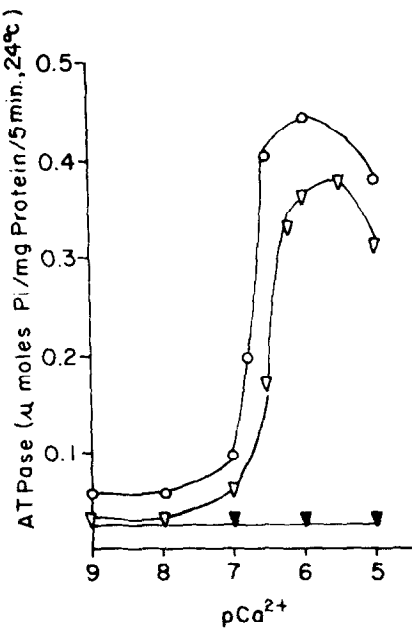

Figure 2. Effect of sodium fluoride on actomyosin ATPase activity. Control actomyosin (Δ-Δ). Actomyosin assayed in the presence of sodium fluoride (▲-▲). Actomyosin pretreated with sodium fluoride removed by washing (O-O).

Actomyosin ATPase activity was measured at various calcium concentrations as follows: The reaction medium (1.0 ml) consisted of 25 mM Tris-HCl, pH 7.6, 5.0 mM sodium azide, 2.5 mM $MgCl_2$, 0.3 to 0.6 mg of natural actomyosin, and 10.0 mM Ca-EGTA buffers. The reaction was initiated with ATP (final concentration was 2.5 mM) and it was terminated after five minutes at room temperature with 1.0 ml of 10% trichloroacetic acid. The solution was kept in ice for ten minutes and centrifuged at 2000 x g for 10 minutes. Inorganic phosphate was determined as described by Fiske and Subba Row (10). Blanks contained the same reagents except the actomyosin was added after the addition of trichloroacetic acid. Protein concentration was measured by the Lowry-method, using bovine serum albumin as standard protein (11). A kapp = 1×10^7 was used to calculate the $[Ca^{2+}]$ of Ca-EGTA buffer (12).

Phosphatase activity present in the natural actomyosin preparations and commercial protein kinase was determined as described by Garen and Levinthal, (13) except the measurements were made under phosphorylation conditions. The reaction medium consists of 10 mM p-nitrophenyl phosphate, 25 mM Tris-HCl, pH 7.6, 5 mM sodium azide, 2.5 mM $MgCl_2$, 0.1 to 0.2 mg of natural actomyosin or 0.05 mg of protein kinase in a total volume of 3.0 ml. The phosphatase activity was measured with and without sodium fluoride (20 mM). The increase in absorbency resulting from hydrolysis of p-nitrophenyl phosphate was measured spectrophotometrically at 410 nm as a function of time.

Natural actomyosin (3.0 mg) was incubated for 120 minutes in 1.0 ml of medium containing 0.3 mg of bovine heart protein kinase, 10^{-5} M cyclic AMP, 5.0 mM sodium azide 2.5 mM $MgCl_2$, 30 mM NaF (γ-32p) ATP (0.1 mM, 1×10^9 cpm). The reaction was stopped with 1.0 ml of 10% trichloroacetic acid. The precipitate was collected by centrifugation at 2000 x g for 10 minutes. The precipitate was dissolved in 0.1 to 0.2 ml of 2N NaOH and the actomyosin was reprecipitated with 1.0 ml of 5% trichloroacetic acid. The resulting precipitate was dissolved in 0.1 to 0.2 ml of 0.2 N NaOH and the solution was neutralized with 1.0 N HCl. Phosphorylated proteins were mixed with an equal volume of 2% sodium docecyl sulfate containing 10 mM β-mercaptoethanol and heated at 80^0 C for two minutes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn (14). About 200 μg of the protein (6000 cpm) was applied to each gel column. The optical densities of the protein bands were recorded on a Gilford automatic densitometer. Molecular weights were determined from a standard graph calibrated by the use of proteins of known molecular weights, such as albumin, ovalbumin, pepsin and myoglobin, respectively.

RESULTS

In the absence of sodium fluoride, treatment of purified guinea pig cardiac natural actomyosin with exogenous protein kinase, Mg^{2+}-ATP, and cyclic AMP had no effect on the actomyosin ATPase (Figure 1). Actomyosin ATPase activity was completely inhibited when assayed in the presence of sodium fluoride. However, there was a complete recovery of actomyosin ATPase upon removal of NaF by washing three times with the reaction medium (Figure 2). In the absence of NaF, phosphatase activities of seven different preparations of natural actomyosin was 0.42 ± 0.06, while that of commercially available protein kinase was 15 ± 0.4 (SEM). In the presence of NaF, there was no detectable phosphatase activity associated with the actomyosin, and the phosphatase activity (1.0 ± 0.19) of the commercially available protein kinase was inhibited 94%.

Washed cardiac actomyosin which had been phosphorylated in the presence of sodium fluoride possessed less ATPase activity at all Ca^{2+} concentrations ($P < 0.005$) (Figure 3). When phosphorylated actomyosin was dephosphorylated with either E. coli alkaline phosphatase or commercial protein kinase, the ATPase activity increased between pCa 5.0 and 6.5 ($P < 0.01$). Sodium docecyl sulfate polyacrylamide gel electrophoresis of phosphorylated actomyosin is shown in Figure 4. Radioactivity (32 p) was incorporated into 210,000 dalton band (myosin heavy chain) and 28,000 dalton band (Troponin-I

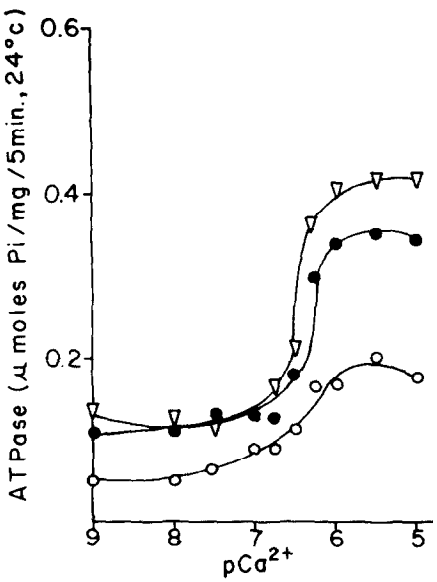

Figure 3. Effect of phosphorylation and dephosphorylation on actomyosin ATPase activity. Actomyosin is phosphorylated with cyclic AMP and protein kinase in the presence of sodium fluoride (0-0). Phosphorylated actomyosin treated with E. coli alkaline phosphatase as described under Methods (Δ-Δ). Phosphorylated actomyosin treated with commercial protein kinase and cyclic AMP as described under Methods (●-●).

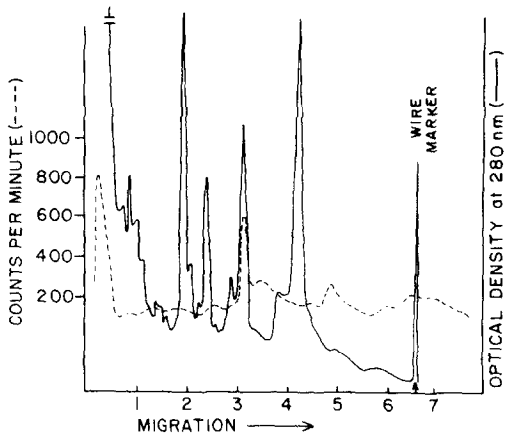

Figure 4. SDS polyacrylamide gel electrophoresis of phosphorylated protein fraction. Phosphorylation was carried out as described in Methods. Electrophoresis was carried out in 10% polyacrylamide gels containing 0.1% SDS. A constant current of 8 mA/tube was applied.

or myosin LC_1), respectively. The other proteins present in the actomyosin complex were 49,000 daltons (actin), 41,000 daltons (Troponin-T), 37,500 daltons (Tropomyosin), and 18,000 daltons (Troponin-C and myosin LC_2).

DISCUSSION

The present study reveals that the phosphorylation of cardiac actomyosin decreases its ATPase activity. However, this result with cardiac actomyosin was obtained only when the natural actomyosin was phosphorylated in the presence of NaF. In the absence of NaF, a result similar to that of Rubio et al (15) was obtained, i.e., there was little change in ATPase activity. Phosphatase activity as measured by the hydrolysis of ρ-nitrophenyl phosphate was found in cardiac natural actomyosin and commercial protein kinase. Both of these phosphatase activities were inhibited by sodium fluoride. When conducting tracer experiments the presence of phosphatase along with actomyosin and protein kinase could actually aid incorporation of label if the protein substrate were already phosphorylated (16). However, when biological or enzymatic activity is the dependent variable, the presence of phosphatase during phosphorylation experiments could lead to erroneous conclusions. When natural actomyosin was incubated with (γ-32p) ATP, most of the label was found in the 28,000 dalton fraction. This would suggest that either myosin subunit LC_1 or troponin-I is phosphorylated. Since LC_1 does not have any influence on actomyosin ATPase and troponin-I has previously been shown to undergo phosphorylation (17), troponin-I is the most likely candidate for phosphorylation. If phosphorylation of troponin-I increases its affinity for actin, one would expect phosphorylation to decrease the ATPase activity of natural actomyosin, since it would decrease the availability of actin for myosin.

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