Vol. 90, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 12, 1979 Pages 694-699

EFFECT OF NOVEL SPECIFIC MYOSIN LIGHT CHAIN KINASE INHIBITORS ON Ca^{2+}-ACTIVATED Mg^{2+}-ATPase OF CHICKEN GIZZARD ACTOMYOSIN

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Received August 15,1979

SUMMARY

Vascular relaxing agents such as N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), N^2-dansyl-L-arginine-4-t-butyl-piperidine amide (No. 233), prenylamine and chlorpromazine that interact with Ca^{2+}-regulated modulator protein of cyclic nucleotide phosphodiesterase inhibited Ca^{2+}-dependent phosphorylation of chicken gizzard myosin light chain. Inhibition by the agents of myosin light chain phosphorylation resulted in inhibition of calcium activated, magnesium dependent adenosine triphosphatase of the gizzard actomyosin. The specificity of these agents for inhibition of light chain phosphorylation was shown by negative effect of these agents on ATPase activity of gizzard actomyosin in the phosphorylated form. Results suggest that the agents provide useful tool for the study on the Ca^{2+}-sensitive regulatory mechanism of modulator-related enzyme systems.

INTRODUCTION

Several drugs that interact with Ca^{2+}-regulated modulator protein (modulator) of cyclic nucleotide phosphodiesterase are found to produce relaxation of smooth muscle tissue such as blood vessel and inhibit interaction between smooth muscle actin and myosin (1, 2, 3). These facts, coupled with the observation that the regulation by Ca^{2+} of smooth muscle actomyosin is based on the phosphorylation of the 20,000 dalton light chain of myosin (4, 5) and this myosin light chain kinase is composed of the modulator protein and a 105,000 dalton component (6), imply that the modulator protein is involved in the regulation of the biological activity of actomyosin. Since the light chain kinase activates the Mg^{2+}-ATPase activity of gizzard actomyosin and as

Abbreviations: W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; SDS, sodium dodecyl sulfate; No. 233, N^2-dansyl-L-arginine-4-t-butyl-piperidine amide

0006-291X/79/190694-06$01.00/0

this is accompanied by a phosphorylation of the actomyosin, it is thought that phosphorylation may be a prerequisite for actin activation. The objective of this communication is to determine using modulator interacting agents whether or not a cause and effect relationship exist between myosin light chain phosphorylation and Ca^{2+}-dependent activation of Mg^{2+}-ATPase of gizzard actomyosin. Our data also suggest that the Ca^{2+}-sensitive regulatory mechanism of gizzard actomyosin is mediated via modulator protein rather than by a troponin-like component.

MATERIALS AND METHODS

Protein preparation - Chicken gizzard actomyosin was prepared according to the method of Sobieszek and Small (7).

Assay for ATPase activity - ATPase activity was determined by liberated inorganic phosphate according to the method of Martin and Dotty (8). The reactions were performed at 25°C in 0.3 ml of reaction mixture containing 0.88 mg/ml to 2 mg/ml gizzard actomyosin, 60 mM KCl, 1 mM cysteine, 40 mM imidazole (pH 7.0 at 25°C), 7 mM $MgCl_2$, 100 μM $CaCl_2$ and 500 μM ATP. After reaction mixtures were pre-equilibrated, the reactions were initiated by addition of ATP and terminated by the addition of 0.7 ml of ice cold 10% trichloroacetic acid.

Phosphorylation analyses - Phosphorylation was determined by incorporated ^{32}P in myosin light chain from [γ - ^{32}P]ATP (3 μCi/assay tube) in the same conditions as for the ATPase activity assay except for the addition of sufficient [γ - ^{32}P]ATP triethylammonium salt (3,000 Ci/mM) to give 50,000 - 100,000 cpm in 5 μl of final mixture. Two methods for analyzing the extent of phosphorylation were employed; 1) The reaction was terminated by the addition of 0.7 ml of ice cold 10% trichloroacetic acid to 0.3 ml of the reaction mixture. The sample was centrifuged at 2,000 rpm for 5 min, the pellet resuspended in ice cold 5% trichloroacetic acid solution and centrifugation-resuspension cycle was repeated three times. The final pellet was dissolved in 1 ml of 0.5 N NaOH and the radioactivity was measured by liquid scintillation counter. 2) The reaction was terminated by adding 0.1 ml of 6% SDS solution to 0.3 ml of the reaction mixture and the solution containing 88 ug protein was applied to SDS polyacrylamide gels. SDS polyacrylamide electrophoresis was carried out at 10% acrylamide and 100 mM sodium phosphate buffer (pH 7.2). After electrophoresis, the gels were stained by coomassie brilliant blue and the profile of protein was recorded by densitometry. The gel was then sliced with a gel slicer into 1 mm sections and each piece of gel was dissolved in 30% hydrogen peroxide solution by heating at 80°C for 3 hr and counted by in a liquid scintillation counter.

RESULT AND DISCUSSION

The distribution of ^{32}P incorporation in gizzard actomyosin was examined by SDS gel electrophoresis. Shown in Fig. 1 is a scan of a stained gel and the ^{32}P content associated with each component. The only subunit with a

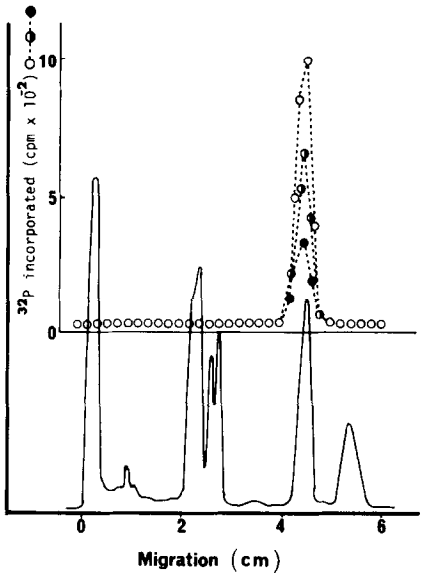
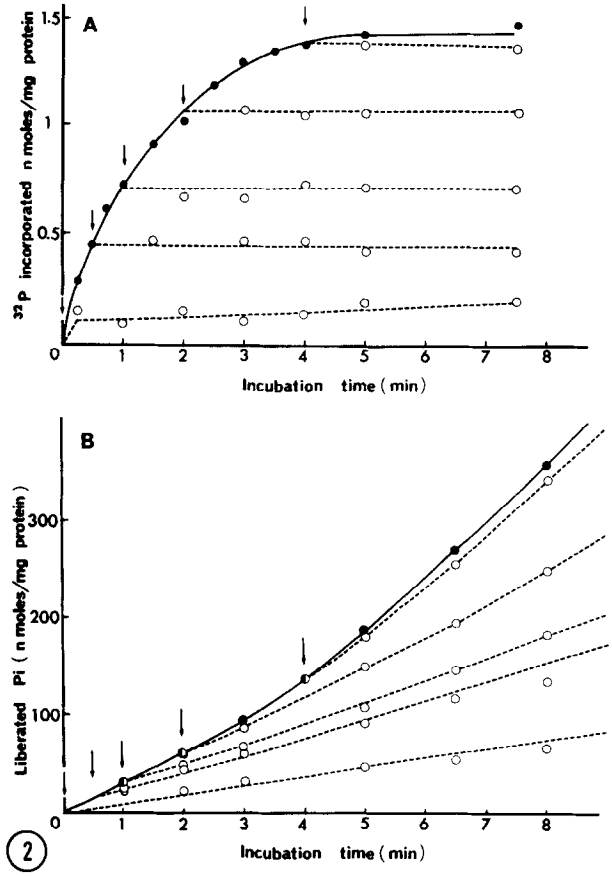


Fig. 1 Density scan of SDS gel of gizzard actomyosin and effect of W-7 on ^{32}P incorporation into 20,000 dalton light chain of gizzard myosin.

The reaction mixture (0.3 ml) contained; 40 mM imidazole (pH 7.0), 1.7 mM $MgCl_2$, 100 μM $CaCl_2$, 1 mM cysteine, 60 mM KCl, 250 μM ATP, [γ - ^{32}P]ATP (7.5 μCi/tube), gizzard actomyosin (2 mg/ml) and various concentrations of W-7 (—○— none, —◑— 50 μM, —●— 100 μM). The reaction was terminated by adding 0.1 ml of 6% SDS solution. Then, 44 μl of this mixture (88 μg actomyosin) was applied to SDS polyacrylamide gel electrophoresis.
—— : density scan of actomyosin, ○ ◑ ● : radioactivity of ^{32}P.

significant extent of ^{32}P incorporation was the 20,000 dalton light chain of myosin which agrees with the results of Gorecka et al. (9).

The ^{32}P content associated with 20,000 dalton light chain of myosin decreased dose-dependently by the addition of W-7 (Fig. 1). The level of ^{32}P association with myosin light chain increased time-dependently and this increase was blocked immediately by addition of W-7 (Fig. 2A). Increase in the extent of ^{32}P incorporation was not observed later than 4 min (Fig. 2A), suggesting that myosin light chain phosphorylation was saturated until 5 min in this experimental condition. The addition of W-7, when it was added later than 4 min, did not affect the level of ^{32}P incorporation, nor ATPase activity. The velocity of actomyosin ATPase activity was directly proportional to the level of ^{32}P association with myosin light chain (Fig. 2B). Not only W-7 but also other modulator interacting agents such as No. 233, prenylamine and



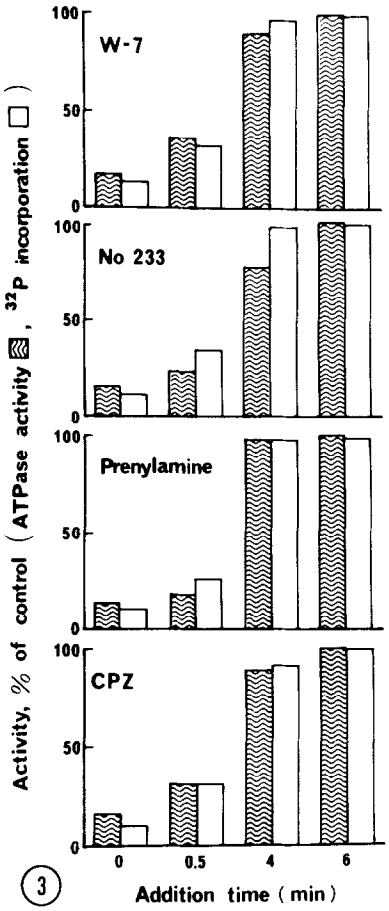


Fig. 2 Effects of W-7 on the phosphate incorporation and the Mg^{2+}-Ca^{2+}-ATPase activity of chicken gizzard actomyosin.

The same assay condition was employed for determination of both ATPase activity and ^{32}P incorporation. The reaction mixture (0.3 ml) contained 40 mM imidazole (pH 7.0), 7 mM $MgCl_2$, 100 μM $CaCl_2$, 1 mM cysteine, 60 mM KCl, 500 μM ATP, and 0.88 mg/ml gizzard actomyosin. The reaction was initiated by adding ATP at 0 min. At the time indicated by the arrow 100 μM W-7 was added to the reaction mixture.
A: Phosphorylation B: ATPase activity
—●—: in the absence of W-7, ·····○·····: in the presence of W-7

Fig. 3 Effect of modulator-interacting agents on the ATPase activity and ^{32}P incorporation of chicken gizzard actomyosin.

ATPase activity and ^{32}P incorporation were assayed under the same condition as described in Fig. 2. W-7(100 μM), No. 233(33 μM), prenylamine (167 μM) and chlorpromazine (CPZ) (83 μM) were added to the reaction mixture at various time indicated in the figure after initiation of the reaction.

chlorpromazine produced inhibition of myosin light chain phosphorylation and Ca^{2+}-Mg^{2+}-ATPase (Fig. 3). These drugs inhibited both myosin light chain phosphorylation and Ca^{2+}-Mg^{2+}-ATPase to the same extent (Fig. 3).

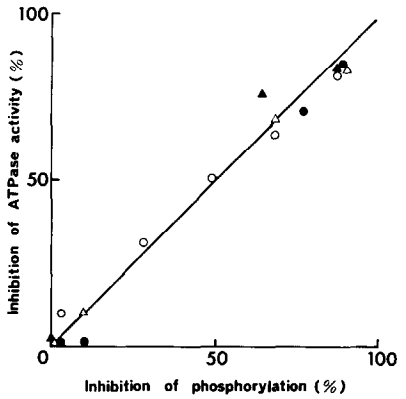


Fig. 4 Correlation between Ca^{2+}-Mg^{2+}-ATPase inhibition and phosphorylation of 20,000 dalton light chain inhibition.
○ : W-7, △:Chlorpromazine, ● :Prenylamine, ▲:No. 233

Significant inhibition by these drugs was not observed when they were added to the reaction mixture later than 4 min after initiation of the reaction (Fig. 2, 3), suggesting that all drugs tested did not affect gizzard actomyosin ATPase in the phosphorylated form and inhibit specifically modulator related enzyme reaction such as phosphorylation of myosin light chain. Figure 4 indicate that the decrease in Ca^{2+}-Mg^{2+}-ATPase activity is directly proportional to the inhibition of myosin light chain phosphorylation. Myosin light chain phosphorylation was reported to take place in the canine heart in vivo (10), as well as in perfused rabbit heart (11) and in the skeletal muscle of live frog (12) or rabbit (13). More recently Bárány et al. (14) reported that light chain phosphorylation is involved in activation of muscle contraction by providing a driving force to place the cross-bridges in the vicinity of actin filaments. A phosphorylation-dephosphorylation of myosin light chain linked to contractile activity of frog muscles raises the question of its relationship to the well established troponin-tropomyosin control of contraction. The calcium dependence of chicken gizzard myosin light chain kinase has been shown to be mediated by a 16,500 dalton calcium-binding subunit (6). In the absence of this component the kinase is inactive. Furthermore, this calcium binding protein has been shown to be identical to moculator protein

of cyclic nucleotide phosphodiesterase. Since modulator interacting agents such as W-7 produced relaxation of vascular smooth muscle (1, 2, 3), modulator protein has been strongly suggested to be involved in control mechanism of actin-myosin interaction. This communication presents the data that such agents that interact with modulator protein and produce relaxation of vascular smooth muscle inhibit indirectly Ca^{2+}-Mg^{2+}-ATPase through direct inhibition of myosin light chain phosphorylation. These agents seem to be specific for inhibition of modulator-catalyzed reaction such as myosin light chain phosphorylation and may provide useful tool for the study of actin-myosin interaction.

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