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# AUTOPHOSPHORYLATION OF β-CONNECTIN (TITIN 2) IN VITRO

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Phosphorylation of β-connectin (titin 2), an elastic protein of chicken breast muscle, occurred in the presence of [$\gamma$-$^{32}$P] ATP, 0.2 mM $CaCl_2$ and 25 mM phosphate buffer, pH 7.0. Addition of 3 mM $MgCl_2$ did not affect the phosphorylation. However, $Ca^{2+}$ ions were required for the phosphorylation and EGTA inhibited it even if $MgCl_2$ were present. Myosin light chain kinase (gizzard MLCK), cAMP dependent protein kinase (A kinase), and protein kinase C (C kinase) did not phosphorylate β-connectin *in vitro* under optimal conditions. Thus it appears that β-connectin, possibly containing a domain homologous with MLCK, has an autophosphorylating action. 

Connectin, also called titin, is a giant elastic protein (MW, ~ 3 million) linking the myosin filament to the Z lines of vertebrate striated muscle sarcomeres (reviewed in 1, 2). Sequence analyses have suggested that there are two types of repeated motifs consisting of about 100 amino acids which are common to immunoglobulin, N-CAM and fibronectin (3, 4). In addition, twitchin, a 668.5 kDa connectin of the nematode, *C.*

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elegans, has a domain homologous with myosin light chain kinase (MLCK) (3).

Somerville and Wang (5) reported that frog skeletal muscle connectin contained approximately 15 moles of bound inorganic phosphate per mole (3 million) and in vivo phosphorylation resulted in phosphorylation of two moles of serine residues per mole of connectin. Furthermore, similar results were obtained with excised mouse diaphragm incubated with $^{32}P$ orthophosphate (6).

In view of in vivo phosphorylation of connectin (5, 6), we have tested in vitro phosphorylation of connectin by several protein kinases without success. However, interestingly, autophosphorylation of connectin has been observed under the conditions where most protein kinases do not act, i. e., in the absence of added $Mg^{2+}$ ions.

## MATERIALS AND METHODS

Preparation of proteins. β-Connectin was purified from chicken breast muscle stored for 12 h at 4°C (7). α-Connectin was prepared from rabbit skeletal muscle according to Kimura and Maruyama (8). Myosin light chain kinase (MLCK) was prepared from chicken gizzard (9). A catalytic subunit of A kinase (bovine heart) was purchased from Sigma. C kinase prepared from rat brain was kindly supplied by Dr. K. Oishi (10).

In vitro phosphorylation. A few milligrams of β-connectin per ml in 0.15 M potassium phosphate buffer (pH 7.0) were first diluted to 0.6 mg/ml with 0.15 M phosphate buffer, and were then made to 0.1 mg/ml in the reaction mixture as below. β-Connectin (0.1 mg/ml) was incubated with 6 μCi/ml of [$\gamma$-$^{32}P$] ATP (Amersham, 6000 Ci/mmole) in a solution consisting of 0.1 mM ATP, 0.2 mM $CaCl_2$ and 25 mM potassium phosphate buffer (pH 7.0). The total volume was 50 μl. After incubation at 25°C, the reaction was terminated by adding 1.5 vol. of an SDS solution (10% SDS, 5% 2-mercaptoethanol and 0.1 M Tris-HCl, pH 6.8).

SDS gel electrophoresis and autoradiography. SDS gel electrophoresis was performed by the method of Laemmli using 2-6% polyacrylamide gels (11). After gels were stained with Coomassie Brilliant Blue, gels were dried on a Whatman No. 5 filter paper. Autoradiography was carried out at -80°C for 7-21 days with Kodak X-omat film and Cornex Lightening-plus intensifying screen (DUPONT).

## RESULTS AND DISCUSSION

Phosphorylation of connectin became apparent when incubated with [$\gamma$-$^{32}$P] ATP for longer than 15 min and became maximal on 1 h incubation (Fig. 1). Phosphorylation was not detected in the absence of 0.2 mM $CaCl_2$. This fact was also confirmed in the experiments shown in Figs. 2 and 3. $Mg^{2+}$ ions, 0.3-3 mM, could not substitute for 0.2 mM $CaCl_2$, but did not affect the action of $Ca^{2+}$ (Fig. 2). These experiments were carried out in a solution containing 25 mM potassium phosphate (pH 7.0). Addition of 50 mM NaCl did not affect the phosphorylation in the presence of 0.1 mM $CaCl_2$ (Fig. 3). However, 3 mM β-mercaptoethanol completely inhibited the phosphorylation even in the presence of 0.2 mM $CaCl_2$. Calmodulin (20 μg/ml), cAMP (1 μM) or cGMP (1 μM) did not affect at all.

Addition of MLCK, A kinase, or C kinase did not result in phosphorylation of connectin. These protein kinases are known to require $Mg^{2+}$ for their activities. Chicken gizzard MLCK is active in the presence of both $Ca^{2+}$ (0.2 mM) and $Mg^{2+}$ (1 mM). However, MLCK did not increase the extent of phosphorylation of connectin in the presence of $Ca^{2+}$ and $Mg^{2+}$. Therefore, it is unlikely that some contaminant protein kinases phosphorylated connectin in the present experiments. In addition, crude connectin and purified connectin did not show any difference in phosphorylation.

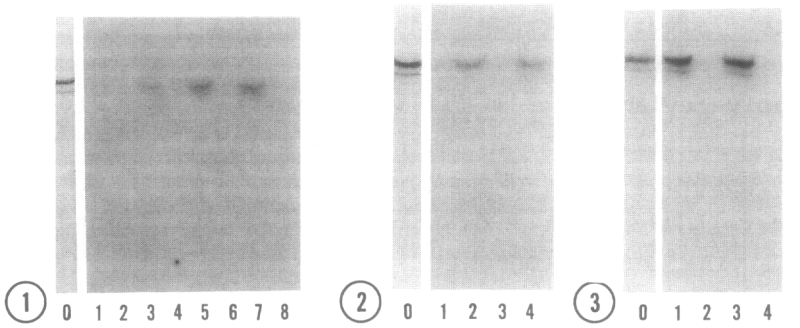


Figure 1. Phosphorylation of β-connectin at varied incubation time. Connectin, 0.1 mg/ml, 0.1 mM ATP containing [$\gamma$-$^{32}$P] ATP, 0.3 mM $MgCl_2$ and 25 mM potassium phosphate buffer, pH 7.0. 0, SDS gel electrophoresis pattern; 1-8, autoradiogram; 0.2 mM $CaCl_2$ (1, 3, 5, 7); 5 mM EGTA (2, 4, 6, 8). Incubated for 5 min (1, 2), 15 min (3, 4), 30 min (5, 6) and 60 min (7, 8).

Figure 2. Effects of $Ca^{2+}$ and $Mg^{2+}$ ions on the phosphorylation of β-connectin. 0, SDS gel electrophoresis pattern; 1-4, autoradiogram; 1, control; 2, 0.2 mM $CaCl_2$; 3, 3 mM $MgCl_2$ + 5 mM EGTA; 4, 0.2 mM $CaCl_2$ + 3 mM $MgCl_2$. Incubated for 1 h. Other conditions, as in Figure 1.

Figure 3. Effects of $Ca^{2+}$ and $Na^{+}$ ions on the phosphorylation of β-connectin. 0, SDS gel electrophoresis pattern; 1-4, autoradiogram; 1, 0.1 mM $CaCl_2$; 2, 2.5 mM EGTA; 3, 0.1 mM $CaCl_2$ + 50 mM NaCl; 4, 2.5 mM EGTA + 50 mM NaCl. Incubated for 1 h. Other conditions, as in Figure 1.

The present work strongly suggests autophosphorylation of β-connectin. The presence of a domain highly homologous with MLCK in the amino acid sequences (3) supports this possibility. Most of the present work were done with β-connectin, a proteolytic product (MW, ~ 2 million) of the mother molecule, α-connectin (MW, ~ 3 million). α-Connectin showed an extent of phosphorylation similar to β-connectin. β-Connectin is easily degraded into 1700 and 400 kDa fragments (7). In the SDS gel electrophoresis patterns of Figs. 1, 2 and 3, the band below β-connectin was 1700 kDa

fragment. This band was also phosphorylated (Fig. 1, 2 and 3). The 1700 kDa fragment has been shown to be located on the myosin filament (Kimura, S., Itoh, Y., Suzuki, T. and Maruyama, K., unpublished). It seems that the site of phosphorylation is located on this less extensible domain of α-connectin (12).

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