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CALCIUM-DEPENDENT PHOSPHORYLATION OF BOVINE CARDIAC C-PROTEIN BY PHOSPHORYLASE KINASE

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Summary. Phosphorylase kinase catalyzed the calcium-dependent phosphorylation of bovine cardiac C-protein. Phosphorylation of C-protein by phosphorylase kinase reached nearly 2 mol [^{32}P]/mol C-protein. Tryptic phosphopeptide mapping and phosphoamino acid analysis indicated that phosphorylase kinase maybe phosphorylating some of the same seryl residues that undergo phosphorylation by cAMP-dependent protein kinase and that C-protein from bovine and chicken heart are structurally different. Bovine cardiac C-protein was not a substrate for a number of calcium and cyclic nucleotide-independent protein kinases, suggesting that phosphorylation of cardiac C-protein is restricted to protein kinases which are modulated by calcium and cAMP.

C-protein, a regulatory component of cardiac myofibrils, undergoes reversible phosphorylation in intact hearts (reviewed in Ref. 1). The protein is phosphorylated in response to cardioactive agents that regulate calcium and cAMP levels in the heart (1-4). In vitro, C-protein can be phosphorylated by purified cAMP-dependent protein kinase (5-7) or by calcium/phospholipid-dependent protein kinase [protein kinase C] (8). It is believed that C-protein regulation of the contractile state of cardiac muscle is modulated by reversible phosphorylation. C-Protein phosphorylated by cAMP-dependent protein kinase is less effective in stimulating myosin ATPase than is nonphosphorylated C-protein (9).

Hartzell and Glass (5) reported that a preparation of C-protein, highly purified from chicken hearts, was contaminated with an uncharacterized calcium/calmodulin-dependent protein kinase which was capable of catalyzing the phosphorylation of C-protein. Since phosphorylase kinase is a calcium/calmodulin-dependent protein kinase (10) and it is known to phosphorylate a number of other myofibril proteins (11-13), we examined the possibility that C-protein may serve as a substrate for phosphorylase kinase.

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METHODS

Enzymes and proteins. Detergent-washed myofibrils were prepared from fresh bovine hearts (14). C-protein was extracted from the myofibrils with 100 mM EDTA/ 124 mM NaH_2PO_4/ 31 mM Na_2HPO_4, pH 5.9. C-Protein was then purified by the procedure previously described for the purification of chicken heart C-protein (5). The C-protein preparation was nearly homogeneous as judged by SDS polyacrylamide gel electrophoresis, and it was free of endogenous calcium-dependent (Fig. 1) or calcium/calmodulin-dependent protein kinases (data not shown) which could phosphorylate C-protein. Phosphorylase kinase is routinely purified in our laboratory according to the procedure of Cohen (15). The purity of the phosphorylase kinase was confirmed by its specificity to phosphorylate glycogen synthase only in site 2 (16). The catalytic subunit of cAMP-dependent protein kinase was purified by the procedure of Reimann and Beham (17). cAMP-dependent protein kinase inhibitor peptide [PKI-(5-24)], was a generous gift of Dr. Edwin Krebs (18). Calcium and cyclic nucleotide-independent protein kinases were prepared as in (16,19). Other materials were prepared as previously described or obtained from sources previously noted (7).

Phosphorylation of C-protein. Phosphorylation of C-protein was carried out at 30°C in a reaction mixture containing 4 μM C-protein/200 μM [γ^{32}P]ATP (200-2000 cpm/pmol)/10 mM magnesium acetate/0.1 mM EDTA/0.05 mM EGTA/2 mM dithiothreitol/30 to 60 mM NaCl/0.1 to 2 mM NaN_3/2.0 μM PKI-(5-24)/0.5 mg bovine serum albumin per ml and the indicated buffer and kinase. Unless otherwise noted the reaction mixture also contained 0.1 mM calcium chloride. The mixture containing all of the reactants except [γ^{32}P]ATP-Mg was preincubated at 30°C for 2 min. The reaction was initiated by the addition of [γ^{32}P]ATP-Mg. The incorporation of ^{32}P into C-protein was determined by spotting an aliquot on Whatman P81 filter paper and processing as previously described (7,20). Stoichiometry of phosphorylation was calculated using a molecular weight of 155,000 for C-protein. Reactions containing phosphorylase kinase were corrected for autophosphorylation. Autophosphorylation accounted for <10% of the total phosphate incorporated.

Other methods. Tryptic digestion and phosphopeptide mapping of ^{32}P-labeled C-protein was carried out as described in (7). Samples for phosphoamino acid analysis were prepared as described in (7) and were separated by high-voltage electrophoresis on TLC cellulose plates at pH 1.9 (21). SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (22). All pHs were determined at the indicated buffer concentrations at 23°C.

RESULTS

Phosphorylation of C-protein by phosphorylase kinase. As shown in Figure 1, purified phosphorylase kinase catalyzed the phosphorylation of C-protein. The phosphorylation was completely calcium-dependent and was not affected by the cAMP-dependent protein kinase inhibitor peptide. Figure 1 (insert) illustrates that the ^{32}P was incorporated into C-protein. Under these reaction conditions, C-protein was phosphorylated at approximately 1/8 the rate of phosphorylase b. When a phosphorylase kinase preparation purified through the Sepharose 4B column (15) was applied to an HPLC DEAE 5PW column (Waters Corp.) and then eluted with a linear gradient, the kinase activity with phosphorylase b or C-protein as substrate co-eluted from the column (data not shown) providing further evidence that C-protein was phosphorylated by phosphorylase kinase

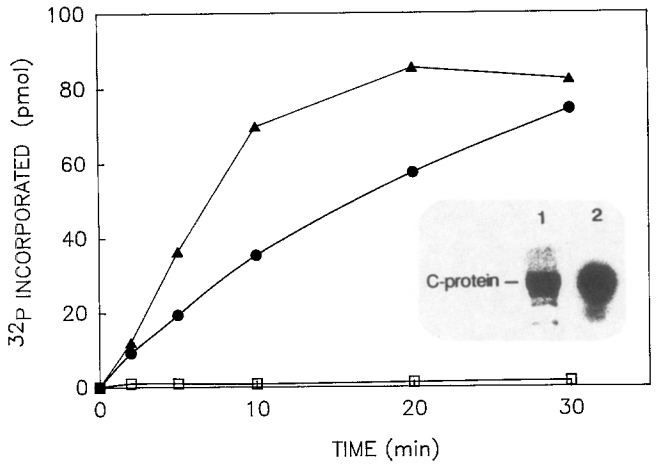

Figure 1. Phosphorylation of C-protein and phosphorylase b by phosphorylase kinase. The incubation was carried out in 25 mM Tris-HCl, pH 7.2, with 4 μM C-protein and 50 μg phosphorylase kinase/ml (●), 4 μM phosphorylase b and 10 μg phosphorylase kinase/ml (▲), or 4 μM C-protein and no added kinase (□). There was no significant phosphorylation of phosphorylase b in the absence of added kinase (data not shown). Insert: SDS-polyacrylamide gel electrophoresis of a sample of C-protein phosphorylated by phosphorylase kinase to 2.0 mol [^{32}P]/mol C-protein. Lane 1, Coomassie blue stained gel, and Lane 2, autoradiogram of the gel. There were no bands in the area of the gel not shown.

and not by a contaminating protein kinase in the phosphorylase kinase preparation. In addition, when the phosphorylase kinase was autophosphorylated to 2.4 mol ^{32}P/mol kinase monomer, the initial rate of C-protein phosphorylation was increased about 2-fold (data not shown) indicating that the C-protein kinase activity, like the phosphorylase kinase activity (23) was increased by autophosphorylation.

The apparent Km of phosphorylase kinase for C-protein was assessed by varying the concentration of C-protein in the reaction mixture. The Km was determined to be 2.7±0.77 μM. This value is lower than the Km reported for phosphorylase b under similar reaction conditions (23), suggesting that low concentrations of C-protein should inhibit phosphorylation of phosphorylase b by phosphorylase kinase. As shown in Table I, C-protein was an effective inhibitor of phosphorylase b phosphorylation.

We examined the possibility that C-protein may serve as a substrate for a number of calcium and cyclic nucleotide-independent protein kinases including casein kinase I, casein kinase II, glycogen synthase kinase 3, and glycogen synthase kinase M. However, these kinases were unable to phosphorylate C-protein at a significant rate (data not shown).

Stoichiometry and peptide mapping of C-protein phosphorylated by phosphorylase kinase and cAMP-dependent protein kinase. As shown in Fig. 2A, phosphorylation of C-protein by the catalytic subunit of cAMP-dependent protein kinase leveled off at about 3 mol ^{32}P/mol C-protein. When C-protein was

TABLE I

EFFECT OF C-PROTEIN ON THE PHOSPHORYLATION OF PHOSPHORYLASE b BY PHOSPHORYLASE KINASE

C-Protein μM	% Inhibition
0	0
1	28
2	42
4	55

The incubation was carried out in 25 mM Tris-HCl, pH 7.2, in a final volume of 50 μl as described in the text. The concentration of phosphorylase kinase was 1 μg/ml, phosphorylase b was 1 μM, and C-protein was at the indicated concentrations. After 5 min incubation, the reactions were terminated by the addition of 25 μl of SDS sample preparation buffer (22), the samples were heated for 3 min at 100°C, and 20 μl aliquots were applied to 7% polyacrylamide gels. After electrophoresis in the presence of SDS, the gels were stained with Coomassie blue, the band containing phosphorylase was cut out, and radioactivity was determined by liquid scintillation spectrometry. The data are presented as % inhibition of phosphorylase b phosphorylation.

phosphorylated by phosphorylase kinase, phosphorylation rapidly approached 1 mol ^{32}P/mol C-protein (Fig. 2B). With prolonged incubation phosphorylation approached 2 mol ^{32}P/mol C-protein (data not shown). When C-protein was phosphorylated by phosphorylase kinase to about 1.4 mol ^{32}P/mol C-protein and then the catalytic subunit of cAMP-dependent protein kinase was added, phosphorylation was increased to about 3 mol ^{32}P/mol C-protein (Fig. 2B). Continued incubation with catalytic subunit did not significantly increase C-protein phosphorylation (data not shown). These results suggested that phosphorylase kinase may be phosphorylating C-protein at some of the same sites phosphorylated by the catalytic subunit. Phosphoamino acid analysis of [^{32}P]C-protein phosphorylated by either kinase revealed that all of the ^{32}P was in phosphoserine (data not shown).

Tryptic phosphopeptide mapping of [^{32}P]C-protein on reversed-phase HPLC revealed that radioactivity incorporated into C-protein by the catalytic subunit was separated into two fractions (Fig. 3A). The first fraction, which was only slightly retarded on the column, contained about 70% of the ^{32}P recovered. The second fraction, which eluted from the column at about 35 min contained the remaining 30%. Assuming an equal recovery of all of the [^{32}P]phosphopeptides from the column (total recovery was about 80%), there were about 1.9 mol ^{32}P/mol C-protein associated with the first fraction and about 0.8 mol associated with the second fraction. Other mapping procedures will be required to determine if the first fraction contains more than one phosphopep-

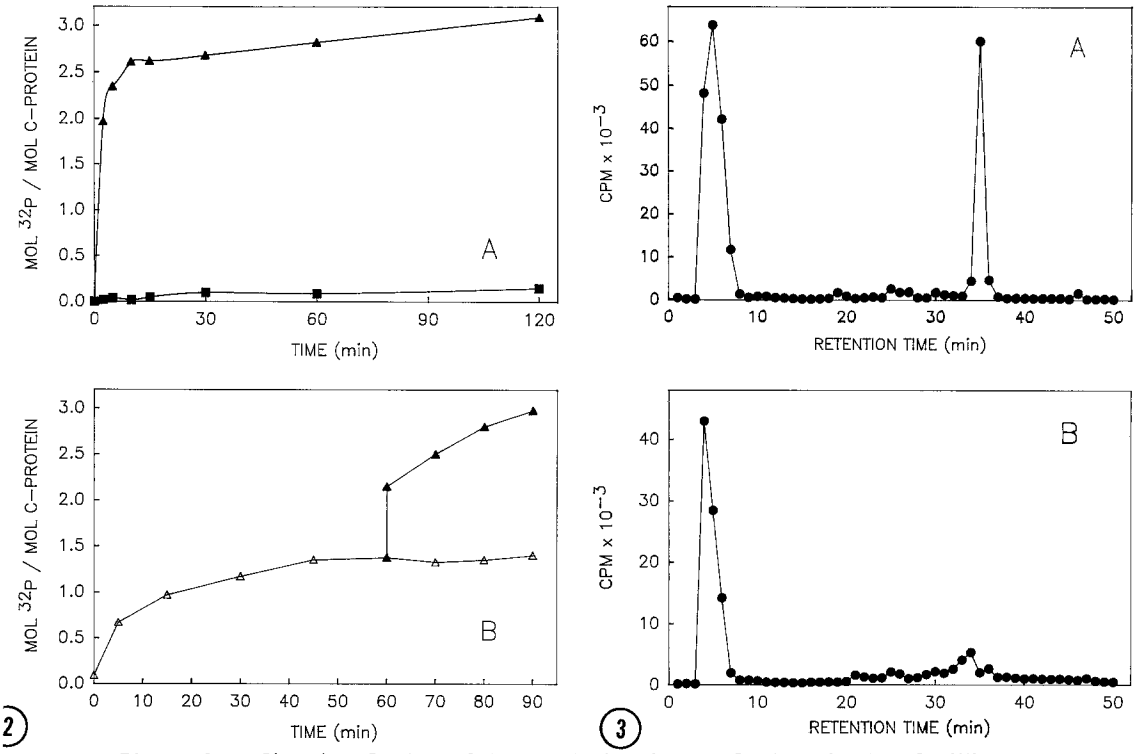

Figure 2. Phosphorylation of C-protein by the catalytic subunit of cAMP-dependent protein kinase and phosphorylase kinase. The reaction was carried out in 25 mM Tris, pH 7.2. In panel A, C-protein was incubated in the absence (■) or in the presence (▲) of the catalytic subunit of cAMP-dependent protein kinase (2 μg/ml) in the absence of calcium. In Panel B, C-protein was incubated with phosphorylase kinase (50 μg/ml) as described in the text (△). After 60 min, EGTA was added to a final concentration of 0.16 mM and incubation was continued in the absence (△) or in the presence (▲) of the catalytic subunit of cAMP-dependent protein kinase (2 μg/ml). Protein kinase inhibitor peptide was omitted from the incubation mixtures.

Figure 3. Reversed phase HPLC chromatograms of [^{32}P]tryptic peptides of C-protein phosphorylated by phosphorylase kinase and the catalytic subunit of cAMP-dependent protein kinase. C-Protein was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase to 2.7 mol ^{32}P/mol C-protein (Panel A) or phosphorylase kinase to 1.4 mol ^{32}P/mol C-protein (Panel B). ^{32}P-Labeled C-protein samples were isolated and tryptic digests were prepared and separated on a Synchropak RP-P C_{18} reversed phase column as previously described (7) except that the column was washed for 5 min with 0.1% trifluoroacetic acid in water at a flow rate of 1 ml/min before applying the linear gradient. Fractions of 1 ml were collected and radioactivity determined by Cerenkov counting.

tide or a single peptide with more than one phosphorylation site. When the tryptic [^{32}P]phosphopeptides obtained from C-protein phosphorylated by phosphorylase kinase were chromatographed, virtually all of the ^{32}P was eluted in the fraction which was only slightly retarded (Fig. 3B).

DISCUSSION

This study demonstrates that phosphorylase kinase catalyzes calcium dependent phosphorylation of bovine heart C-protein. Phosphorylation by

phosphorylase kinase appears to involve some of the same seryl sites which are phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. This was not unexpected since phosphorylase kinase shares some of the requirements for site specificity with that of cAMP-dependent protein kinase (23). In vivo phosphorylation of cardiac C-protein is known to be influenced by cardioactive agents that regulate the levels of cAMP and calcium in the heart (1). We found that bovine heart C-protein was not phosphorylated by several calcium and cyclic nucleotide-independent protein kinases. These results indicate that the phosphorylation of C-protein is, at least thus far, restricted to calcium-dependent and cyclic nucleotide-dependent protein kinases. The results presented in this study and those reported previously (7) should allow us to define the protein kinases and phosphatases responsible for phosphorylating and dephosphorylating C-protein in vivo. These studies should also make it possible to design experiments to better understand the exact role of C-protein phosphorylation in the regulation of the contractile state of heart muscle.

C-Protein from different species and muscle types have apparent molecular weights ranging from 130,000 to 165,000 and it has been suggested that these represent various isoforms of C-protein (1). In this regard, it is of interest to note that the tryptic peptide map obtained from bovine heart C-protein phosphorylated by the catalytic subunit of cAMP-dependent protein kinase is quite different from the pattern we previously observed when chicken heart C-protein was phosphorylated by the same kinase (7). In the latter, three tryptic [^{32}P]phosphopeptide fractions of about equal amounts designated T1, T2, and T3 eluted at 7 min, 14 min, and 38 min respectively (7). Chicken heart C-protein peptide T3 and the second tryptic peptide fraction from bovine heart C-protein elute from the column at about the same place. However, T3 from chicken heart C-protein contains exclusively phosphothreonine. There was no significant phosphothreonine in bovine heart C-protein phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Therefore, these results also provide direct evidence for structural differences in bovine and chicken cardiac C-protein.

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