Phosphorylation of skeletal-muscle troponin I and troponin T by phospholipid-sensitive Ca^{2+} -dependent protein kinase and its inhibition by troponin C and tropomyosin

Gonzalo J. MAZZEI and J. F. KUO

Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

(Received 25 July 1983/Accepted 31 October 1983)

Skeletal-muscle troponin I and troponin T were found to be rapidly phosphorylated by cardiac phospholipid-sensitive Ca^{2+} -dependent protein kinase, with K_m values of 6.66 and 0.13 μM respectively. Stoichiometric phosphorylation of skeletal troponin I (endogenous phosphate content 0.7 mol/mol) indicated that the Ca^{2+} -dependent enzyme and cyclic AMP-dependent protein kinase incorporated 0.9 and 0.8 mol/mol respectively. The same experiments with skeletal troponin T (endogenous phosphate content 1.9 mol/mol) revealed a maximal phosphorylation of 2 mol/mol by the Ca^{2+} -dependent enzyme, whereas the cyclic AMP-dependent enzyme was unable to phosphorylate troponin T. The Ca^{2+} -dependent enzyme phosphorylated both serine and threonine residues in skeletal and cardiac troponin I or troponin T; the cyclic AMP-dependent enzyme, in comparison, phosphorylated only serine in skeletal and cardiac troponin I. Although an equimolar amount of skeletal or cardiac troponin C markedly inhibited (80–90%) phosphorylation of skeletal and cardiac troponin I by the Ca^{2+} -dependent enzyme, these troponin C preparations inhibited only phosphorylation of skeletal troponin I, but not that of cardiac troponin I, by the cyclic AMP-dependent enzyme. Calmodulin and Ca^{2+} -binding protein S-100a could mimic the inhibitory effect of troponin C. A tissue specificity appeared to exist for the skeletal troponin T–skeletal troponin C interaction. Inhibition of troponin T phosphorylation by an equimolar amount of troponin C was lower than that of troponin I phosphorylation; these findings might explain in part why troponin T was the major substrate for the Ca^{2+} -dependent enzyme in the troponin complex. The present studies indicate that skeletal and cardiac troponin I and troponin T were effective substrates for phospholipid-sensitive Ca^{2+} -dependent protein kinase, suggesting a potential involvement of this Ca^{2+} -effector enzyme in the regulation of myofibrillar activity.

Contraction of skeletal and cardiac muscle is mediated by an interaction of Ca^{2+} with the tropomyosin–troponin complex that regulates actomyosin ATPase (Ebashi *et al.*, 1969; Weber & Murray, 1973). Following the first report by Bailey & Villar-Palasi (1971) that cyclic AMP-dependent protein kinase phosphorylated skeletal troponin I, investigations into the phosphorylation of components in tropomyosin–troponin have been performed in many laboratories (Stull *et al.*, 1972; Perry & Cole, 1974; Stull & Buss, 1977; Katoh *et al.*, 1983). Enzymes that phosphorylate cardiac and/or skeletal troponin I include phosphorylase *b* kinase (Perry & Cole, 1974; Cole & Perry, 1975), cyclic AMP-dependent protein kinase (Bailey & Villar-Palasi, 1971; Cole & Perry, 1975), cyclic

GMP-dependent protein kinase (Blumenthal *et al.*, 1978; Lincoln & Corbin, 1978) and phospholipid-sensitive Ca^{2+} -dependent protein kinase (Katoh *et al.*, 1983). Enzymes identified to date that phosphorylate skeletal and/or cardiac troponin T are phosphorylase *b* kinase (Perry & Cole, 1974; Moir *et al.*, 1977), troponin T kinase (Gusev *et al.*, 1980; Villar-Palasi & Kumon, 1981), casein kinase TS (Pinna *et al.*, 1981), and phospholipid-sensitive Ca^{2+} -dependent protein kinase (Katoh *et al.*, 1983). Possible functional roles of troponin phosphorylation in muscle contraction are not fully understood. It is suggested that such a phosphorylation reaction is involved in the speed and/or force of contraction (England, 1975; Solaro *et al.*, 1976; Moir & Perry, 1980; Moir *et al.*, 1980), the

negative-feedback regulation of contraction related to the Ca^{2+} affinity of the troponin complex (Holroyde *et al.*, 1979; Robertson *et al.*, 1982; Yamamoto & Ohtsuki, 1982), a long-term modulation of cardiac contractility (Villar-Palasi & Kumon, 1981; Katoh *et al.*, 1983), and proteolysis of troponin (Toyo-Oka, 1982).

We reported previously (Katoh *et al.*, 1983) that cardiac troponin I and troponin T, present as the free subunits or in complex forms as troponin and tropomyosin-troponin, were rapidly phosphorylated by phospholipid-sensitive Ca^{2+} -dependent protein kinase purified from bovine heart extracts (Wise *et al.*, 1982). The Ca^{2+} -dependent enzyme appears to have a greater ability, compared with other protein kinases, to phosphorylate cardiac troponin subunits, in terms of broader substrate specificity, lower K_m values, higher $V_{\max}$ values and/or higher stoichiometric incorporation of phosphate (Katoh *et al.*, 1983). In the present studies, we report phosphorylation of skeletal troponin subunits by the Ca^{2+} -dependent enzyme and regulation of phosphorylation of skeletal and cardiac troponin I and troponin T by skeletal and cardiac troponin C and tropomyosin.

Experimental

Materials

Phosphatidylserine (bovine brain), phosphoserine, phosphotyrosine, phosphothreonine and histone H1 (lysine-rich histone, type III-S) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; calmodulin (homogeneous) from bovine brain was a product of Sciogen Inc., Detroit, MI, U.S.A.

Methods

Phospholipid-sensitive Ca^{2+} -dependent protein kinase was purified (80–95% homogeneous) from bovine heart (Wise *et al.*, 1982). Cyclic AMP-dependent protein kinase was partially purified (Shoji *et al.*, 1977) and its catalytic subunit purified to homogeneity (Bechtel *et al.*, 1977), both from bovine heart. Tropomyosin-troponin complex, troponin complex and the troponin subunits (C, I and T) were purified from fresh rabbit mixed skeletal muscle and frozen bovine heart by the methods of Potter (1982). Tropomyosin from bovine heart (Stull & Buss, 1977) and rabbit skeletal muscle (Greaser & Gergely, 1973) were purified as described in the references. The α - and β -isoforms of tropomyosin were prepared from the non-modified fully reduced tropomyosin, as described by Smillie (1982). The Ca^{2+} -binding protein S-100a was purified from bovine brain (Isobe *et al.*, 1977). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared as

described by Post & Sen (1967). Protein was determined by the method of Bradford (1976).

The assay conditions for the Ca^{2+} -dependent enzyme were essentially the same as those described previously (Kuo *et al.*, 1980; Wise *et al.*, 1982). Briefly, the standard reaction mixture contained, in a final volume of 0.2 ml, 5 μmol of Pipes (1,4-piperazinediethanesulphonic acid) (pH 6.5), 2 μmol of MgCl_2 , 5 μg of phosphatidylserine, 0.04 μmol of EGTA, with or without 0.1 μmol of CaCl_2 . Cyclic AMP-dependent protein kinase was assayed as described by Shoji *et al.* (1977); the assay mixture contained 5 μmol of potassium phosphate buffer (pH 7), 2 μmol of MgCl_2 and 20 pmol of cyclic AMP. The amounts of the enzymes and substrates were indicated in the legends of the individual Figures and Tables. The enzyme reaction was started by adding $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (containing $3 \times 10^6\text{--}6 \times 10^6$ c.p.m.). The concentration of ATP used was 100 μM , to maximize protein phosphorylation (Katoh *et al.*, 1983). All incubations were performed in duplicate, with assay errors being less than 5%. KCl (50 mM) was included in the incubation mixtures to prevent or minimize precipitation of contractile proteins.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and radioautography of the phosphorylated proteins were performed as described previously (Wrenn *et al.*, 1980; Katoh *et al.*, 1981, 1983). Endogenous phosphate content of skeletal troponin I and troponin T was determined by the method of Itaya & Ui (1966) after washing the proteins three times with 10% (v/v) trichloroacetic acid and ashing by the method of Ames (1966). Phosphoamino acids were analysed by high-voltage electrophoresis on cellulose plates (Eastman Chemical Co., Rochester, NY, U.S.A.) at pH 1.9 and 3.5 as described by Hunter & Sefton (1980). The phosphoamino acids were detected by spraying the plates with 0.2% (w/v) ninhydrin dissolved in 95% (v/v) ethanol.

Results

A survey of endogenous substrates of a high-salt (0.6 M-KCl) extract of the ether-dried powder of rabbit skeletal muscle for the phospholipid-sensitive Ca^{2+} -dependent protein kinase revealed the phosphorylation of proteins of M_r 38000 and 23000, with relative mobilities similar to those of skeletal troponin T and troponin I respectively. To support these preliminary findings further, skeletal troponin subunits were purified and found to be phosphorylated by phospholipid-sensitive Ca^{2+} -dependent protein kinase from bovine heart, as described in the Experimental section.

Phospholipid-sensitive Ca^{2+} -dependent protein

Fig. 1. Phosphorylation of rabbit skeletal troponin T and troponin I by cardiac phospholipid-sensitive Ca^{2+} -dependent and cyclic AMP-dependent protein kinases

Troponin I (30 μ g) and troponin T (30 μ g) were incubated with the enzymes as described in the Experimental section. (a) Troponin T stained with Coomassie Brilliant Blue; (b) radioautography of troponin T; (c) troponin I stained with Coomassie Brilliant Blue; (d) radioautography of troponin I. The amount of troponin T and troponin I applied was 5 μ g/lane. The separating gel was 12% acrylamide containing 0.1% sodium dodecyl sulphate. Abbreviations: sTn-T, skeletal troponin T; sTn-I, skeletal troponin I; PS, phosphatidylserine; PL-Ca-PK, phospholipid-sensitive Ca^{2+} -dependent protein kinase; A-PK, cyclic AMP-dependent protein kinase.

kinase phosphorylated the isolated skeletal troponin T (Fig. 1b) or troponin I (Fig. 1d), whereas cyclic AMP-dependent protein kinase could only phosphorylate troponin I. It was also found, by using the troponin complex as substrate, that the Ca^{2+} -dependent enzyme phosphorylated both troponin T and I subunits, whereas the cyclic AMP-dependent enzyme was unable to phosphorylate either (results not shown).

Exhaustive phosphorylations of the skeletal troponin I revealed that both the Ca^{2+} -dependent and cyclic AMP-dependent enzymes incorporated 0.9 and 0.8 mol of ^{32}P /mol of subunit respectively (Fig. 2a). The endogenous phosphate content of skeletal troponin I (0.70 ± 0.10 mol/mol; mean $\pm$ S.E.M. for ten determinations) is within the range of values (0.4–0.9) reported for skeletal troponin I isolated from fresh muscle by using troponin C affinity chromatography (Cole & Perry, 1975). Therefore it appeared that there were at least two phosphorylation sites in troponin I. Although the cyclic AMP-dependent enzyme was unable to phosphorylate skeletal troponin T, the Ca^{2+} -dependent enzyme incorporated 2 mol of ^{32}P /mol of the protein (Fig. 2b). If one considers the endogenous phosphate content of troponin T, which was determined to be 1.98 ± 0.14 mol/mol (six determinations), the potential phosphorylation sites in skeletal troponin T for the Ca^{2+} -dependent enzyme could be as high as four. The endogenous phosphate content in the skeletal troponin T seen

in the present studies was higher than the values (0.7–0.8) reported by others (Huang *et al.*, 1974; Moir *et al.*, 1974; Perry & Cole, 1974; Lallemand *et al.*, 1975). The differences may be due to the methods of preparation and the history of the tissue used (Cole & Perry, 1975; Solaro *et al.*, 1976).

The apparent K_m values of the Ca^{2+} -dependent protein kinase for skeletal troponin T and troponin I were found to be $0.13 \pm 0.06 \mu M$ (three determinations) and $6.7 \pm 0.9 \mu M$ (three determinations) respectively, whereas the V_{max} value for the phosphorylation of troponin T was about 60% of that of troponin I. The apparent K_m of the cyclic AMP-dependent enzyme for troponin I, in comparison, was $33 \pm 2 \mu M$ (three determinations).

Analysis of the phosphoamino acids indicated that the Ca^{2+} -dependent enzyme preferentially phosphorylated the serine residues, rather than the threonine residues, in the skeletal troponin complex (Fig. 3, lane 1) and skeletal troponin I (lane 4). The enzyme, on the other hand, preferentially phosphorylated the threonine residues in cardiac troponin T (lane 3), but phosphorylated both residues in skeletal troponin T (lane 2) and cardiac troponin I (lane 5) at similar rates. The cyclic AMP-dependent enzyme phosphorylated only troponin I (Fig. 1), and this phosphorylation occurred only at the serine residues for both skeletal and cardiac troponin I (Fig. 3, lanes 6 and 7). The electrophoretic separations of phosphoamino acids shown in Fig. 3 were performed at pH 1.9, which

Fig. 2. Stoichiometry of phosphorylation of isolated skeletal troponin I and troponin T by cardiac phospholipid-sensitive Ca^{2+} -dependent and cyclic AMP-dependent protein kinases

(a) Troponin I ($2.75 \mu\text{g}$ or 0.12 nmol) or (b) troponin T ($0.71 \mu\text{g}$ or 0.019 nmol) was incubated with either phospholipid-sensitive Ca^{2+} -dependent protein kinase ($2 \mu\text{g}$; ●) in the presence of phosphatidylserine ($5 \mu\text{g}$) and CaCl_2 ($500 \mu\text{M}$) or with the catalytic subunit of cyclic AMP-dependent protein kinase ($0.8 \mu\text{g}$; ○). Other conditions were as given in the Experimental section. The data was corrected for the blank values seen in the absence of added troponin and in the absence of added enzymes. The M_r values of troponin I and troponin T used for calculation were 20699 and 30522 respectively.

can effectively separate phosphoserine and phosphothreonine (Hunter & Sefton, 1980). To verify that phosphotyrosine was not present in the phos-

Fig. 3. Phosphoamino acids in phosphorylated skeletal and cardiac troponin components

Skeletal and cardiac troponin preparations were incubated with phospholipid-sensitive Ca^{2+} -dependent protein kinase (PL-Ca-PK, $0.2 \mu\text{g}$; lanes 1–5) or the catalytic subunit of the cyclic AMP-dependent protein kinase (A-PK, $0.8 \mu\text{g}$; lanes 6 and 7). The products of acid hydrolysis were subjected to electrophoresis toward the anode on cellulose thin-layer plates at pH 1.9. The radioautograms shown were taken collectively from several experiments performed under the same conditions. In all cases, the acid hydrolysis of the phosphorylated proteins was performed for various durations (1–3 h) and the ^{32}P -labelled phosphoamino acids were identified with the aid of standards. Abbreviations: sTn, skeletal troponin complex; sTn-T, skeletal troponin T; cTn-T, cardiac troponin T; sTn-I, skeletal troponin I; cTn-I, cardiac troponin I; Thr-P, phosphothreonine; Ser-P, phosphoserine; P_i , phosphate.

phothreonine spots, the troponin samples were electrophoresed at pH 3.5, a condition under which the two phosphoamino acids can be clearly separated (Hunter & Sefton, 1980); the results clearly indicated that the tyrosine residues were not phosphorylated (results not shown).

The decomposition of phosphoserine and phosphothreonine in 6M-HCl for 2 h was reported by Bylund & Huang (1976) to be 30% and 8% respectively. Assuming the percentage decomposition to be the same on every point in Fig. 4, it is possible to state that the Ca^{2+} -dependent protein kinase phosphorylates preferentially threonine rather than serine in cardiac troponin T (Fig. 4a), reaching equal extents of phosphorylation after 2 h of incubation with the enzyme. In skeletal troponin I, however, serine is definitely the preferential site for the Ca^{2+} -dependent kinase (Fig. 4b). These observations support the qualitative findings in Fig. 3.

Phosphorylation of skeletal (Fig. 5a) and cardiac troponin I (Fig. 5b) by the Ca^{2+} -dependent enzyme was progressively and markedly inhibited

by cardiac or skeletal troponin C. Although nearly complete inhibition of skeletal troponin I phosphorylation was noted at a molar ratio (troponin C/troponin I) of 2:1, the inhibition of cardiac troponin I phosphorylation remained at about 80% at a molar ratio of 3:1. As reported previously by others (Cole & Perry, 1975), phosphorylation by cyclic AMP-dependent protein kinase of skeletal troponin I was inhibited (Fig. 5a), whereas that of cardiac troponin I was not inhibited (Fig. 5b), by troponin C of both skeletal and cardiac origins.

In addition to troponin C, we found that other homologous Ca^{2+} -binding proteins, such as calmodulin and Ca^{2+} -binding protein S-100a, at a molar ratio of 1:1, similarly inhibited phosphorylation of cardiac and skeletal troponin I by the Ca^{2+} -dependent enzyme (Table 1). Calmodulin, like troponin C, had little effect on histone phosphorylation; a more marked inhibition (42–56%) seen for S-100a could be attributed to a strong interaction between histone H1, a basic protein, and S-100a, a strongly acidic protein (Isobe *et al.*, 1977).

Although a complete inhibition of skeletal troponin-T phosphorylation by the Ca^{2+} -dependent enzyme was noted for skeletal troponin C at a molar ratio of 3:1 with respect to the substrate, a maximum of about 50% inhibition was noted for

cardiac troponin C (Fig. 6a). For cardiac troponin-T phosphorylation by the enzyme, the maximal inhibition attainable by either cardiac or skeletal troponin C was only 20–30% (Fig. 6b).

Skeletal tropomyosin was found to inhibit, to various extents, phosphorylation of troponin preparations by the Ca^{2+} -dependent protein kinase (Fig. 7). At a molar ratio of 6:1 with respect to different troponin preparations, for example, skeletal tropomyosin inhibited by about 50–70% the phosphorylation of skeletal troponin complex or skeletal and cardiac troponin T, compared with inhibition by about 30–40% of phosphorylation of skeletal and cardiac troponin I. Phosphorylation of histone H1 by the Ca^{2+} -dependent enzyme or that of skeletal and cardiac troponin I by the cyclic AMP-dependent enzyme, in comparison, was not affected by tropomyosin. Similar results were observed with cardiac tropomyosin (results not shown).

The ability of tropomyosin to interact with troponin T and to inhibit phosphorylation of this tropomyosin-binding subunit of troponin was further investigated. No appreciable difference was found between the α - and β -isoforms of skeletal or cardiac tropomyosin in their ability to inhibit phosphorylation of skeletal or cardiac troponin T by the Ca^{2+} -dependent protein kinase.

Table 1. *Comparative effects of Ca^{2+} -binding proteins on the phosphorylation of histone H1 and of cardiac and skeletal troponin I by cardiac phospholipid-sensitive Ca^{2+} -dependent protein kinase*

Histone H1 (11 μg , 0.55 nmol), cardiac troponin I (11 μg , 0.4 nmol) and skeletal troponin I (20 μg , 0.87 nmol) were incubated for 5 min with the enzyme as described in the Experimental section. The Ca^{2+} -binding proteins were present at the indicated molar ratios. Data shown are means $\pm$ S.E.M. for three experiments. The values in parentheses are percentages of controls.

Addition and molar ratio to substrate	Protein kinase activity (pmol of P incorporated/min)		
	Histone H1	Cardiac troponin I	Skeletal troponin I
None (control)	8.61 $\pm$ 0.45 (100)	7.06 $\pm$ 0.14 (100)	1.44 $\pm$ 0.12 (100)
Calmodulin			
1:1	9.25 $\pm$ 0.31 (106)	1.04 $\pm$ 0.07 (15)	0.38 $\pm$ 0.13 (27)
2:1	7.03 $\pm$ 0.22 (81)	0.72 $\pm$ 0.10 (10)	0.25 $\pm$ 0.03 (18)
3:1	5.76 $\pm$ 0.10 (66)	0.70 $\pm$ 0.06 (10)	0.15 $\pm$ 0.03 (11)
S-100a			
1:1	3.82 $\pm$ 0.16 (44)	0.91 $\pm$ 0.09 (13)	0.11 $\pm$ 0.01 (8)
2:1	4.71 $\pm$ 0.10 (54)	0.70 $\pm$ 0.01 (10)	0.08 $\pm$ 0.01 (6)
3:1	5.04 $\pm$ 0.19 (58)	0.42 $\pm$ 0.07 (6)	0 $\pm$ 0 (0)
Skeletal troponin C			
1:1	8.70 $\pm$ 0.21 (101)	1.12 $\pm$ 0.05 (16)	0.18 $\pm$ 0.02 (13)
2:1	7.04 $\pm$ 0.20 (81)	0.84 $\pm$ 0.09 (12)	0.15 $\pm$ 0.01 (11)
3:1	7.33 $\pm$ 0.21 (84)	0.70 $\pm$ 0.01 (10)	0.11 $\pm$ 0.03 (8)
Cardiac troponin C			
1:1	6.61 $\pm$ 0.29 (76)	2.04 $\pm$ 0.07 (29)	0.48 $\pm$ 0.07 (34)
2:1	7.50 $\pm$ 0.27 (87)	1.47 $\pm$ 0.01 (21)	0.43 $\pm$ 0.06 (30)
3:1	8.42 $\pm$ 0.29 (97)	1.40 $\pm$ 0.12 (20)	0.23 $\pm$ 0.08 (16)

Fig. 4. Comparative rates of phosphorylation of serine and threonine residues in troponin subunits

(a) Cardiac troponin T (30 µg) and (b) skeletal troponin I (20 µg) were incubated with phospholipid-sensitive Ca^{2+} -dependent protein kinase (0.2 µg) for different time intervals as indicated. The phosphoamino acids were separated as in Fig. 3. Spots that co-migrated with the respective standard phosphoamino acids (phosphoserine and phosphothreonine) were scraped from the plate and the radioactivity was counted. ○, Phosphothreonine; ●, phosphoserine.

Discussion

The present studies clearly show that skeletal troponin I and troponin T are effective substrates for phospholipid-sensitive Ca^{2+} -dependent protein kinase, findings similar to those reported previously for the same troponin subunits from cardiac muscle (Katoh *et al.*, 1983).

We observed that both serine and threonine residues in skeletal troponin I and troponin T were phosphorylated by the Ca^{2+} -dependent enzyme, whereas only serine in skeletal troponin I was phosphorylated by cyclic AMP-dependent protein

kinase (Fig. 3). Others (Moir & Perry, 1977, 1980; Perry, 1980; Risnik *et al.*, 1980; Pinna *et al.*, 1981) have reported that serine in skeletal and cardiac troponin I and troponin T is the major site of phosphorylation by other protein kinases. It appears therefore that the Ca^{2+} -dependent enzyme had a greater phosphorylation capacity and that threonine was specifically phosphorylated by this enzyme. Although the stoichiometric phosphorylation of skeletal troponin I by the Ca^{2+} -dependent enzyme revealed that only 0.9 mol of ^{32}P was incorporated/mol of protein (Fig. 2a), other lines of evidence indicated that two sites were likely to be phosphorylated by the enzyme. These lines of evidence include (a) both serine and threonine were phosphorylated (Fig. 3) and (b) endogenous phosphate content was determined to be 0.70 mol/mol. Following the same reasoning, it seems that there are also two sites in cardiac troponin I that could be potentially phosphorylated by the enzyme, because the enzyme (a) phosphorylated both serine and threonine residues (Fig. 3), and (b) incorporated 1.7 mol of ^{32}P /mol of the subunit, which already contained 0.3 mol of endogenous phosphate/mol of the protein (Katoh *et al.*, 1983).

The Ca^{2+} -dependent enzyme incorporated 2 mol of ^{32}P /mol of skeletal troponin T (Fig. 2b), findings similar to those for cardiac troponin T (Katoh *et al.*, 1983). If one considers the endogenous phosphate content of 1.9 mol/mol of skeletal troponin T (the present paper) and 0.4 mol/mol of cardiac troponin T (Katoh *et al.*, 1983), this number of sites that could be potentially phosphorylated by the Ca^{2+} -dependent enzyme could be four and three for the skeletal and the cardiac protein respectively. It should be noted here that cyclic AMP-dependent protein kinase was not able to phosphorylate troponin T. Troponin T kinase probably is the only other enzyme identified that can effectively phosphorylate this troponin subunit (Gusev *et al.*, 1980; Villar-Palasi & Kumon, 1981). It remains to be seen whether the multiple phosphorylation sites in troponin T for the Ca^{2+} -dependent enzyme overlap with the single phosphorylation site (i.e. the N-terminal serine residue) reported for troponin T kinase by Gusev *et al.* (1980).

It has been reported that phosphorylation by cyclic AMP-dependent protein kinase of skeletal troponin I was completely inhibited by an equimolar amount of troponin C, but this inhibition was not observed for cardiac troponin I (Cole & Perry, 1975). In comparison, phosphorylation of both skeletal and cardiac troponin I by phosphorylase b kinase (which phosphorylated the protein very poorly) was inhibited by troponin C (Perry & Cole, 1974; Cole & Perry, 1975). The present studies (Fig. 5), although confirming their find-

Fig. 5. Effect of skeletal and cardiac troponin C on the phosphorylation of skeletal and cardiac troponin I by cardiac phospholipid-sensitive Ca^{2+} -dependent and cyclic AMP-dependent protein kinases

Cardiac phospholipid-sensitive Ca^{2+} -dependent protein kinase (PL-Ca-PK, $0.2\mu\text{g}$) and cyclic AMP-dependent protein kinase (A-PK, $0.8\mu\text{g}$) were used to phosphorylate (a) skeletal troponin I ($30\mu\text{g}$) and (b) cardiac troponin I ($11\mu\text{g}$) in the presence of various molar ratios of cardiac ($\circ$, Δ) or skeletal ($\bullet$, $\blacktriangle$) troponin C, as indicated. The activity values for the phosphorylation of skeletal or cardiac troponin I by the enzymes in the absence of added troponin C were taken as 100%. The abbreviations used were the same as in Figs. 1 and 3. Phosphorylation by PL-Ca-PK (—) or A-PK (---).

Fig. 6. Differential effect of skeletal and cardiac troponin C on the phosphorylation of skeletal and cardiac troponin T by the cardiac phospholipid-sensitive Ca^{2+} -dependent protein kinase

Cardiac phospholipid-sensitive Ca^{2+} -dependent protein kinase ($0.2\mu\text{g}$) was used to phosphorylate (a) skeletal troponin T ($30\mu\text{g}$) and (b) cardiac troponin T ($29\mu\text{g}$) in the presence of various molar ratios of troponin T to cardiac ($\circ$) and skeletal ($\bullet$) troponin C, as indicated. The enzyme-activity values for the phosphorylation of skeletal or cardiac troponin T in the absence of added troponin C were taken as 100%. The abbreviations used were the same as in Figs. 1 and 3.

ings regarding the cyclic AMP-dependent enzyme, suggest that the characteristic of troponin I phosphorylation by the Ca^{2+} -dependent enzyme was similar to that by phosphorylase b kinase. The differential effect of troponin C, as mentioned above, was consistent with the observations that the cyclic AMP-dependent enzyme could not phosphorylate troponin I in the skeletal troponin

complex but it could do so in that from cardiac muscle (Katoh *et al.*, 1983). Although troponin C could markedly inhibit phosphorylation of skeletal and cardiac troponin I by the Ca^{2+} -dependent enzyme (Fig. 5), the remaining (10–20%) activity seen in the presence of an equimolar amount of troponin C may account for the phosphorylation seen for the troponin I subunit in skeletal troponin

Fig. 7. Effect of skeletal tropomyosin on the phosphorylation of cardiac and skeletal troponin preparations and histone H1 by the cardiac phospholipid-sensitive Ca^{2+} -dependent and cyclic AMP-dependent protein kinases

Skeletal troponin complex ($\circ$, $21\mu\text{g}$), skeletal troponin T (Δ , $20\mu\text{g}$), cardiac troponin T ($\square$, $20\mu\text{g}$), skeletal troponin I ($\bullet$, $17\mu\text{g}$), cardiac troponin I ($\blacktriangle$, $5.5\mu\text{g}$) and histone H1 ($\blacksquare$, $5.5\mu\text{g}$) were phosphorylated with cardiac phospholipid-sensitive Ca^{2+} -dependent protein kinase (—; $0.2\mu\text{g}$) or cyclic AMP-dependent protein kinase (---; $0.8\mu\text{g}$). Skeletal tropomyosin (sTm) was added to various molar ratios, as indicated. The phosphorylation rates of the individual substrate proteins seen in the absence of added tropomyosin for both enzymes were taken as 100%. The abbreviations used were the same as in Figs. 1 and 3.

complex (results not shown) and cardiac troponin complex (Katoh *et al.*, 1983).

Troponin C was found to inhibit phosphorylation by the Ca^{2+} -dependent enzyme of skeletal troponin T to a greater extent than that of the cardiac troponin T (Fig. 6). It was also noted that phosphorylation of skeletal troponin T by the enzyme was more sensitive to inhibition by skeletal troponin C than by the cardiac troponin C (Fig. 6). These findings, when taken collectively, seem to suggest a tissue specificity for the troponin T-troponin C interactions. Such a tissue specificity, however, seems to be less pronounced for the troponin I-troponin C interactions (Fig. 5), or the interactions involving troponin I or troponin T with tropomyosin (Fig. 7). Phosphorylase *b* kinase slowly phosphorylated skeletal troponin T, and this reaction was shown to be nearly completely inhibited by an equimolar amount of skeletal troponin C (Perry & Cole, 1974). The varying degree of inhibition (20–60%) of phosphorylation seen in the present studies under the similar condition (Fig. 6) suggested that the characteristic of troponin T phosphorylation by the Ca^{2+} -dependent enzyme and phosphorylase *b* kinase, unlike troponin I phosphorylation by the two enzymes, may be quite different.

Tropomyosin has been shown to interact with troponin T (Jackson *et al.*, 1975; Pearlstone & Smillie, 1983) and troponin I (Pearlstone & Smillie, 1983). The site(s) of interaction of troponin T with tropomyosin are located within amino acid residues 1–158 in the presence of Ca^{2+} (Jackson *et al.*, 1975; Pearlstone & Smillie, 1983). This peptide (fragment T1) contains one phosphorylation site (serine-1) for troponin T kinase (Perry, 1980; Risnik *et al.*, 1980) and two phosphorylation sites (serine-149 and serine-156) for phosphorylase *b* kinase (Moir *et al.*, 1977; Perry, 1980). In the present studies we found that an equal amount of skeletal tropomyosin inhibited by about 50% the skeletal troponin T phosphorylation by the Ca^{2+} -dependent protein kinase (Fig. 7). It is conceivable, therefore, that one of the multiphosphorylation sites in troponin T for the enzyme (Fig. 2b) may be located at fragment T2 (residues 159–259), which is not masked by interaction of troponin T with tropomyosin. Because serine and threonine were both phosphorylated by the Ca^{2+} -dependent enzyme (Fig. 3) and phosphorylation sites (serine-1, -149 and -156) for other enzymes are located in fragment T1, it is speculated that threonine residues at positions 171, 207, 213, 248 or 249 in fragment T2 (Pearlstone *et al.*, 1976) could be phosphorylated by the Ca^{2+} -dependent enzyme. Perry & Cole (1974) reported that tropomyosin also decreased phosphorylation of troponin T by phosphorylase *b* kinase. Because of the variable effect, they concluded that this inhibition may be due to precipitation resulting from tropomyosin-troponin T interaction. This possibility, however, seems to be eliminated in the present studies, because we found that the tropomyosin inhibition was highly reproducible and remained constant even at a high molar ratio of tropomyosin to troponin T of 6:1, and no precipitates were detected during the course of incubation. Furthermore, the inhibitory effect of tropomyosin on the phosphorylation of troponin I presented here is further evidence for their interaction, as suggested by Pearlstone & Smillie (1983).

There are several protein kinases that phosphorylate troponin. It is likely that phosphorylase *b* kinase is not important *in vivo*, because the rate of troponin I phosphorylation by this enzyme is very slow (Stull, 1980), and phosphorylation of troponin T was observed even in the I strain of mice lacking phosphorylase *b* kinase activity (Moir *et al.*, 1977). Cyclic AMP-dependent protein kinase can phosphorylate only troponin I (Perry & Cole, 1974; Katoh *et al.*, 1983), whereas troponin T kinase can phosphorylate only troponin T (Villar-Palasi & Kumon, 1981). Findings from our previous (Katoh *et al.*, 1983) and present studies clearly indicated that phospholipid-sensitive

Ca^{2+} -dependent protein kinase, compared with other protein kinases, had the greatest capability to phosphorylate the troponin subunits. The total activity in muscle for the Ca^{2+} -dependent enzyme is 5–8-fold lower than cyclic AMP-dependent enzyme activity (Kuo *et al.*, 1980) and 2–3-fold higher than troponin T kinase activity (Gusev *et al.*, 1980; Villar-Palasi & Kumon, 1981). Therefore, since the Ca^{2+} -dependent enzyme activity is perhaps too low to phosphorylate troponin subunits during acute muscle contraction, the role of this phosphorylation may be related to relatively long-term effects. Identification of phosphorylation sites for the Ca^{2+} -dependent enzyme and determination of effects of phosphorylation on interactions of contractile protein components and, ultimately, on the Ca^{2+} -sensitivity and activity of actomyosin ATPase, may yield new insights into the regulatory role by this multifunctional phospholipid/ Ca^{2+} -effector enzyme in the regulation of muscular contractility.

This work was supported by U.S. Public Health Service Grants HL-15696 and NS-17608, and a Muscular Dystrophy Association Postdoctoral Fellowship (G. J. M.).

References

- Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118
- Bailey, C. & Villar-Palasi, C. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 1147
- Bechtel, P. J., Beavo, J. A. & Krebs, E. G. (1977) *J. Biol. Chem.* **252**, 2691–2697
- Blumenthal, D. K., Stull, J. T. & Gill, G. N. (1978) *J. Biol. Chem.* **253**, 334–336
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Bylund, D. B. & Huang, T.-S. (1976) *Anal. Biochem.* **73**, 477–486
- Cole, H. A. & Perry, S. V. (1975) *Biochem. J.* **149**, 525–533
- Ebashi, S., Endo, M. & Ohtsuki, I. (1969) *Q. Rev. Biophys.* **2**, 351–384
- England, P. L. (1975) *FEBS Lett.* **50**, 57–60
- Greaser, M. L. & Gergely, J. (1973) *J. Biol. Chem.* **248**, 2125–2133
- Gusev, N. B., Dobrovolskii, A. B. & Severin, S. E. (1980) *Biochem. J.* **189**, 219–226
- Holroyde, M. J., Howe, E. & Solaro, R. J. (1979) *Biochim. Biophys. Acta* **586**, 63–69
- Huang, T.-S., Bylund, D. B., Stull, J. T. & Krebs, E. G. (1974) *FEBS Lett.* **42**, 249–252
- Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1311–1315
- Isobe, T., Nakatima, T. & Okuyama, T. (1977) *Biochim. Biophys. Acta* **494**, 222–232
- Itaya, K. & Ui, M. (1966) *Clin. Chim. Acta* **14**, 361–366
- Jackson, P., Amphlett, G. W. & Perry, S. V. (1975) *Biochem. J.* **151**, 85–97
- Katoh, N., Wise, B. C., Wrenn, R. W. & Kuo, J. F. (1981) *Biochem. J.* **198**, 199–205
- Katoh, N., Wise, B. C. & Kuo, J. F. (1983) *Biochem. J.* **209**, 189–195
- Kuo, J. F., Andersson, R. G. G., Wise, B. C., Macklerlova, L., Salomonsson, I., Brackett, N. L., Katoh, N., Shoji, M. & Wrenn, R. W. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7039–7043
- Lallemant, C., Seraydarian, K., Mommoerts, W. F. H. M. & Suh, M. (1975) *Arch. Biochem. Biophys.* **169**, 367–371
- Lincoln, T. M. & Corbin, J. D. (1978) *J. Biol. Chem.* **253**, 337–339
- Moir, A. J. G. & Perry, S. V. (1977) *Biochem. J.* **167**, 333–343
- Moir, A. J. G. & Perry, S. V. (1980) *Biochem. J.* **191**, 547–554
- Moir, A. J. G., Wilkinson, J. M. & Perry, S. V. (1974) *FEBS Lett.* **42**, 253–256
- Moir, A. J. G., Cole, H. A. & Perry, S. V. (1977) *Biochem. J.* **161**, 371–382
- Moir, A. J. G., Solaro, R. J. & Perry, S. V. (1980) *Biochem. J.* **185**, 505–513
- Pearlstone, J. R. & Smillie, L. B. (1983) *J. Biol. Chem.* **258**, 2534–2542
- Pearlstone, J. R., Carpenter, M. R., Johnson, P. & Smillie, L. B. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1902–1906
- Perry, S. V. (1980) in *Muscle Contraction: Its Regulation Mechanism* (Ebashi, S., Ohtsuki, I. & Endo, M., eds.), pp. 207–220, Japan Scientific Societies Press, Tokyo, and Springer-Verlag, Berlin
- Perry, S. V. & Cole, H. A. (1974) *Biochem. J.* **141**, 733–743
- Pinna, L. A., Meggio, F. & Dediukina, M. M. (1981) *Biochem. Biophys. Res. Commun.* **100**, 449–454
- Post, R. L. & Sen, A. K. (1967) *Methods Enzymol.* **10**, 773–775
- Potter, J. D. (1982) *Methods Enzymol.* **85**, 241–263
- Risnik, V. V., Dobrovolskii, A. B., Gusev, N. B. & Severin, S. E. (1980) *Biochem. J.* **191**, 851–854
- Robertson, S. P., Johnson, D. J., Holroyde, M. J., Kranias, E. G., Potter, J. D. & Solaro, R. J. (1982) *J. Biol. Chem.* **257**, 260–263
- Shoji, M., Patrick, J. G., Davis, C. W. & Kuo, J. F. (1977) *Biochem. J.* **161**, 213–221
- Smillie, L. B. (1982) *Methods Enzymol.* **85**, 234–241
- Solaro, R. J., Moir, A. J. G. & Perry, S. V. (1976) *Nature (London)* **262**, 615–617
- Stull, J. T. (1980) *Adv. Cyclic Nucleotide Res.* **13**, 39–93
- Stull, J. T. & Buss, J. E. (1977) *J. Biol. Chem.* **252**, 851–857
- Stull, J. T., Brostrom, C. O. & Krebs, E. G. (1972) *J. Biol. Chem.* **247**, 5272–5274
- Toyo-Oka, T. (1982) *Biochem. Biophys. Res. Commun.* **107**, 44–50
- Villar-Palasi, C. & Kumon, A. (1981) *J. Biol. Chem.* **256**, 7409–7415
- Weber, A. & Murray, J. M. (1973) *Physiol. Rev.* **53**, 612–673
- Wise, B. C., Raynor, R. L. & Kuo, J. F. (1982) *J. Biol. Chem.* **257**, 8481–8488
- Wrenn, R. W., Katoh, N., Wise, B. C. & Kuo, J. F. (1980) *J. Biol. Chem.* **255**, 12042–12046
- Yamamoto, K. & Ohtsuki, I. (1982) *J. Biochem. (Tokyo)* **91**, 1669–1677