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Phosphorylation of cardiac troponin inhibitory subunit (troponin I) and tropomyosin-binding subunit (troponin T) by cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase

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Cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase phosphorylated cardiac troponin inhibitory subunit (troponin I) and tropomyosin-binding subunit (troponin T), present either as the free form or as the troponin–tropomyosin complex. Exhaustive phosphorylation of troponin I and of troponin T revealed that 1.7 and 2 mol of phosphate was incorporated/mol of the subunits respectively. Cyclic AMP-dependent protein kinase, though incorporating 0.8 mol of phosphate/mol of troponin I, was unable to phosphorylate troponin T. Phosphorylation of troponin I (apparent $K_m = 3.4\,\mu M$; $V_{max.} = 2.6\,\mu$mol/min per mg of enzyme) or troponin T (apparent $K_m = 0.3\,\mu M$; $V_{max.} = 0.5\,\mu$mol/min per mg of enzyme) by the Ca^{2+}-dependent enzyme was inhibited by various agents, such as adriamycin, palmitoylcarnitine, trifluoperazine, melittin and *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide (compound W-7). Ca^{2+} antagonists (such as verapamil), forskolin and ouabain were ineffective. These findings indicate that troponin I and troponin T were effective substrates for this species of Ca^{2+}-dependent protein kinase, suggesting its potential regulatory role in the contractile activity of myofibrils modulated by troponin.

Cardiac troponin, the Ca^{2+}-regulatory protein of myofibrils, is a phosphoprotein with its phosphate content found in the inhibitory subunit (troponin I) and the tropomyosin-binding subunit (troponin T) (Cole & Perry, 1975; Solaro *et al.*, 1976; England, 1976; Moir & Perry, 1977; Stull & Buss, 1977; Moir *et al.*, 1980). Troponin I was phosphorylated when hearts were perfused with β-adrenergic agonists (Solaro *et al.*, 1976; England, 1976; Moir *et al.*, 1980) or when troponin or troponin–tropomyosin preparations were incubated *in vitro* with cyclic AMP-dependent (Cole & Perry, 1975; Moir & Perry, 1977; Stull & Buss, 1977; Blumenthal *et al.*, 1978; Lincoln & Corbin, 1978) or cyclic GMP-dependent (Blumenthal *et al.*, 1978; Lincoln & Corbin, 1978) protein kinase. Although an association between troponin I phosphorylation and contractile-force development does exist, a precise causal relationship between the biochemical reaction and the physiological event appears to be lacking (Solaro *et al.*, 1976; England, 1976; Moir *et al.*, 1980). Katz (1979) has discussed possible effects on troponin I phosphorylation by cyclic AMP-dependent protein kinase on the properties of the cardiac contractile proteins. Much less is known about the phosphorylation of cardiac troponin T and significance of this reaction. Kopp & Barany (1979) observed that Ca^{2+} stimulated phosphorylation of a 38000-M_r protein (presumably troponin T), in addition to troponin I, tropomyosin and myosin P light chain, in perfused rat hearts. More recently, Villar-Palasi & Kumon (1981) reported that purified cardiac troponin T kinase incorporated up to 0.8 mol of phosphate/mol of cardiac troponin T. Gusev *et al.* (1980) have reported that skeletal-muscle troponin T kinase phosphorylated only the *N*-terminal serine residue of skeletal-muscle troponin T.

Both troponin I and troponin T have multiple sites that would be potentially phosphorylated by various protein kinases. Indeed, the total phosphate content of cardiac troponin I has been reported to be 2–3 mol/mol of the subunit (Cole & Perry, 1975; Solaro *et al.*, 1976). Furthermore, phosphorylase

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kinase has been shown to phosphorylate skeletal muscle troponin I and troponin T up to 2 and 3 mol/mol of the subunits respectively (Perry & Cole, 1974), and to phosphorylate cardiac-muscle troponin I about 0.7–1.2 mol/mol (Moir & Perry, 1980). This enzyme, however, on the basis of its low phosphorylation rate (Stull, 1980) and findings made with I strain (lacking phosphorylase kinase) of mice (Moir *et al.*, 1977), is unlikely to be involved in their phosphorylation *in vivo*. It has been shown that serine-20 in troponin I was phosphorylated in hearts perfused with adrenergic agonists (Solaro *et al.*, 1976; England, 1976; Moir *et al.*, 1980) or by cyclic nucleotide-dependent protein kinases (Moir & Perry, 1977; Stull & Buss, 1977; Blumenthal *et al.*, 1978; Lincoln & Corbin, 1978); troponin T was not phosphorylated under the same conditions. These observations clearly suggest the involvements of other species of protein kinases in myofibrillar protein phosphorylation. In the present paper we report an effective phosphorylation of both cardiac troponin I and troponin T by phospholipid-sensitive Ca^{2+}-dependent protein kinases, which we recently purified to near homogeneity from bovine heart extracts (Wise *et al.*, 1982*a*).

Experimental

Materials

Phosphatidylserine (bovine brain), adriamycin, DL-palmitoylcarnitine, ouabain and melittin (bee venom) were from Sigma Chemical Co., St. Louis, MO, U.S.A., and forskolin was from Calbiochem–Behring, La Jolla, CA, U.S.A. Compound W-7 [*N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide] was generously given by Dr. H. Hidaka, Department of Pharmacology, Mie University School of Medicine, Tsu, Japan, or purchased from Rikaken Co., Nagoya, Japan. Trifluoperazine dihydrochloride was kindly provided by Dr. P. T. Ridley of Smith, Kline and French, Philadelphia, PA, U.S.A. Various Ca^{2+} antagonists (Table 2) were kindly provided by Dr. Arnold Schwartz, Department of Pharmacology and Cell Biophysics, University of Cincinnati, Cincinnati, OH, U.S.A.

Methods

Phospholipid-sensitive Ca^{2+}-dependent protein kinase (Wise *et al.*, 1982*a*) was purified (about 95% homogeneous) over 15000-fold, and cyclic AMP- and cyclic GMP-dependent protein kinases (Shoji *et al.*, 1977) were partially purified, all from bovine heart extracts, as we described previously. Myosin light-chain kinase was purified from bovine heart extracts by the calmodulin-affinity method of Walsh *et al.* (1979). Troponin–tropomyosin complex, troponin I and troponin T were purified by the method of Stull & Buss (1977), except that the step of Bio-Gel A-0.5m chromatography was replaced by two consecutive chromatographies on Sepharose 4B. Myosin light chains (unfractionated) were prepared from bovine hearts by the method of Perrie & Perry (1970). [γ-^{32}P]ATP was prepared by the method of Post & Sen (1967). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Incubation conditions for phospholipid-sensitive Ca^{2+}-dependent protein kinase (Kuo *et al.*, 1980; Wrenn *et al.*, 1980) or cyclic nucleotide-dependent protein kinase (Shoji *et al.*, 1977) were the same as described elsewhere, except that the concentration of [γ-^{32}P]ATP (containing 3×10^6–6×10^6 c.p.m.) was increased from 5 to 100 μM in order to maximize phosphorylation of proteins; specific additions to the incubation systems are indicated in the legends to individual Figures and Tables. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and radioautography of phosphorylated proteins were performed as we described previously (Wrenn *et al.*, 1980; Katoh *et al.*, 1981*a*). Myosin light-chain kinase was assayed as reported by Walsh *et al.* (1979). The endogenous phosphate content in the cardiac troponin I, troponin T and troponin–tropomyosin preparations was determined by the method of Itaya & Ui (1966), after ashing of the proteins by the method of Ames (1966).

Results and discussion

Experiments with cardiac troponin–tropomyosin complex as substrate revealed that cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase phosphorylated both troponin I and troponin T (Fig. 1, comparing lanes 1–6), whereas cardiac cyclic AMP-dependent protein kinase phosphorylated only troponin I (Fig. 1, comparing lanes 7–9). There was no detectable endogenous phosphorylation of troponin–tropomyosin complex in the presence of phosphatidylserine plus Ca^{2+} (lane 6) or cyclic AMP (lane 9) without the added enzymes. Likewise there was no detectable endogenous phosphorylation of the Ca^{2+}-dependent enzyme in the presence of phosphatidylserine plus Ca^{2+} (lane 5) or of the cyclic AMP-dependent enzyme in the presence of cyclic AMP without the added troponin–tropomyosin complex (results not shown). In comparison, the calcium-binding subunit (troponin C) and tropomyosin (other components of the troponin–tropomyosin complex) and actin (a slight contaminant of the preparation of the complex) were not phosphorylated by either protein kinases. Phosphorylations of troponin I and troponin T by the Ca^{2+}-dependent enzyme and of troponin I by the cyclic AMP-dependent enzyme were confirmed in experiments with the respective subunits as substrates (Fig. 2), indicating that the presence of other

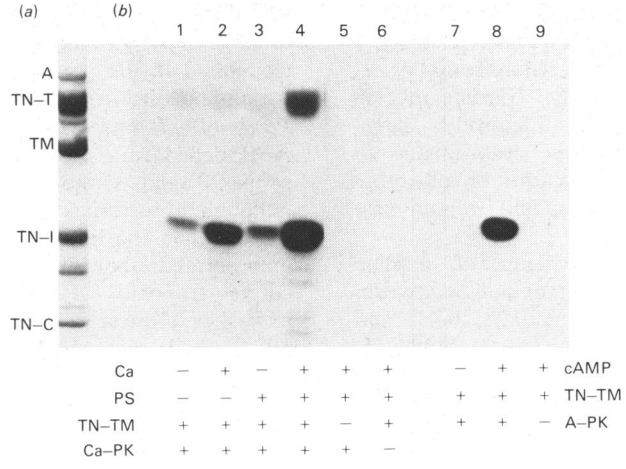

Fig. 1. *Phosphorylations of cardiac troponin–tropomyosin complex by the cardiac phospholipid-sensitive Ca^{2+}-dependent and cyclic AMP-dependent protein kinases*

The complex (100 μg) was incubated in 0.2 ml at 30°C for 5 min with the Ca^{2+}-dependent protein kinase (Ca-PK, 0.02 μg) in the presence or absence of phosphatidylserine (PS, 5 μg) or $CaCl_2$ (500 μM), or with cyclic AMP-dependent protein kinase (A-PK, 30 μg) in the presence or absence of cyclic AMP (cAMP) (0.5 μM). (*a*) Proteins stained with Coomassie Brilliant Blue; (*b*) radioautograph. The separating gel used was 10% acrylamide containing 0.1% sodium dodecyl sulphate. The amount of troponin–tropomyosin complex (TN-TM) applied was 30 μg/lane. Key: A, actin; TM, tropomyosin; TN-C, TN-I and TN-T, troponins C, I and T.

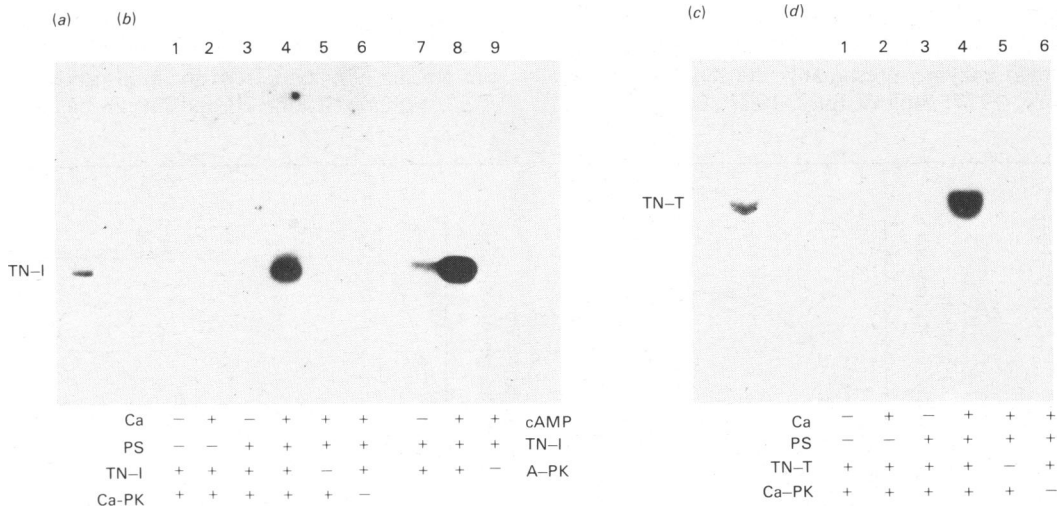

Fig. 2. *Phosphorylation of cardiac troponin I and troponin T by cardiac phospholipid-sensitive Ca^{2+}-dependent and cyclic AMP-dependent protein kinases*

Troponin I (30 μg) and troponin T (30 μg) were incubated with the enzymes as described in Fig. 1 and as indicated. (*a*) Troponin I gel stained with Coomassie Brilliant Blue; (*b*) radioautograph of troponin I phosphorylation; (*c*) troponin T gel stained with Coomassie Brilliant Blue; (*d*) radioautograph of troponin T phosphorylation. The amount of troponin I and troponin T applied was 5 μg/lane. The gel and abbreviations used were the same as in Fig. 1.

component proteins in the troponin–tropomyosin complex did not inhibit the ability of the two protein kinases to phosphorylate their substrates. Cole & Perry (1975) have reported that phosphorylation of cardiac troponin I by the cyclic AMP-dependent enzyme was not inhibited by the addition of troponin C, whereas the phosphorylation of skeletal-muscle troponin I by the same enzyme was inhibited.

Cardiac cyclic GMP-dependent protein kinase was able to phosphorylate troponin I (results not shown), as reported previously by others (Blumenthal *et al.*, 1978; Lincoln & Corbin, 1978). Cardiac myosin light-chain kinase, though effectively phosphorylating myosin light chain, was unable to phosphorylate troponin I, troponin T or any other component proteins in the troponin–tropomyosin complex (results not shown).

Endogenous phosphate contents of cardiac troponin I, troponin T and troponin–tropomyosin complex were determined to be 0.28, 0.41, and 0.64 mol/mol of the proteins respectively. Stoicheiometric studies, with troponin I (Fig. 3*a*) and troponin T (Fig. 3*b*) as substrates, showed that phospholipid-sensitive Ca^{2+}-dependent protein kinase incorporated about 1.7 and 2 mol of ^{32}P/mol of the subunits respectively. On the other hand, cyclic AMP-dependent protein kinase incorporated about 0.8 mol of ^{32}P/mol of troponin I (Fig. 3*a*) without significantly phosphorylating troponin T (Fig. 3*b*). These findings indicated that there were at least two phosphorylation sites in both troponin I and troponin T for the Ca^{2+}-dependent enzyme, compared with perhaps one site on troponin I for the cyclic AMP-dependent enzyme. It appeared that one of the two phosphorylation sites on troponin I for the Ca^{2+}-dependent enzyme was the same as the major site phosphorylated by the cyclic AMP-dependent enzyme, presumably the serine-20 (Moir & Perry, 1977; Stull & Buss, 1977; Blumenthal *et al.*, 1978; Lincoln & Corbin, 1978), because the total ^{32}P incorporation was still about 2 mol/mol of troponin I in the presence of both protein kinases (results not shown). It should be noted that Moir & Perry (1977) have reported previously that cyclic AMP-dependent protein kinase phosphorylated serine-20 and serine-146 of cardiac troponin I, although the former was phosphorylated more rapidly than the latter. Cardiac troponin T kinase incorporated about 0.8 mol of phosphate/mol of cardiac troponin T (Villar-Palasi & Kumon, 1981) and skeletal-muscle troponin T (Perry & Cole, 1974). It is not clear to what extent the two phosphorylation sites in cardiac troponin T for the present Ca^{2+}-dependent enzyme are related to the phosphorylation sites seen with other enzymes.

Kinetic studies on the phosphorylation of cardiac troponin I and troponin T were conducted. The results are summarized and compared with the results obtained with other protein kinases previously reported by others (Table 1). Although the apparent K_m ($3.4 \pm 0.9\,\mu$M, mean ± S.E.M. for three determinations) for troponin I phosphorylation by the Ca^{2+}-dependent enzyme was higher than the values of 0.6 and 0.3 μM that we have reported respectively for histone Hl and myelin basic protein phosphorylation by the same enzyme, the $V_{max.}$ ($2.6 \pm 0.3\,\mu$mol/min per mg of enzyme, three determinations) was higher than the value of 0.8 μmol/min per mg of enzyme seen for both proteins (Wise *et al.*, 1982*b*). Histone Hl and myelin basic protein

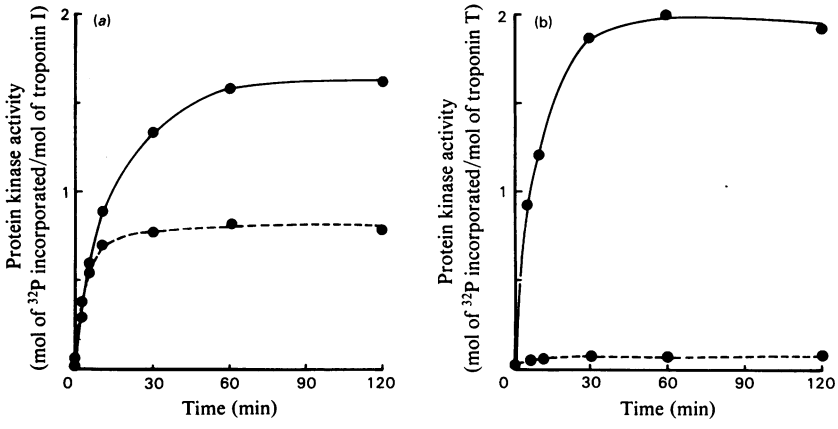

Fig. 3. *Stoicheiometry of phosphorylation of isolated cardiac troponin I or troponin T by cardiac phospholipid-sensitive Ca^{2+}-dependent and cyclic AMP-dependent protein kinases*

(*a*) Troponin I (4.7 μg or 0.17 nmol) or (*b*) troponin T (1.71 μg or 0.043 nmol) was incubated either with the Ca^{2+}-dependent enzyme (——, 0.2 μg) in the presence of phosphatidylserine (5 μg/0.2 ml) and $CaCl_2$ (500 μM) or with the cyclic AMP-dependent enzyme (– – – –, 60 μg) in the presence of cyclic AMP (0.5 μM). Other incubation conditions were the same as those described in the Experimental section. The data presented were corrected for the blank values seen in the absence of added enzyme for the Ca^{2+}-dependent enzyme, or those seen in the absence of added troponin I or troponin T for the cyclic AMP-dependent enzyme. The molecular weights used for calculating stoicheiometry were 27000 for troponin I and 40000 for troponin T.

are by far the two most effective substrate proteins for the nearly homogeneous or homogeneous phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart (Wise *et al.*, 1982*b*) or spleen (Schatzman *et al.*, 1983). The apparent K_m ($0.3 \pm 0.1\,\mu M$, three determinations) and $V_{max.}$ ($0.5 \pm 0.1\,\mu mol/min$ per mg of enzyme, three determinations) for troponin T phosphorylation by the Ca^{2+}-dependent enzyme, however, were similar to the respective values obtained for histone and myelin basic protein mentioned above. It is clear now that troponin I and troponin T can be added to the list of best substrates for the cardiac phospholipid-sensitive Ca^{2+}-dependent enzyme. In terms of K_m, troponin I was an inferior substrate for cyclic nucleotide-dependent protein kinases than for the Ca^{2+}-dependent enzyme (Table 1). Cardiac troponin T appeared to be a better substrate for the Ca^{2+}-dependent enzyme than for troponin T kinase (Table 1), an enzyme selectively phosphorylating the *N*-terminal serine residue of troponin T (Villar-Palasi & Kumon, 1981). Although appropriate studies on cardiac proteins are lacking, phosphorylase kinase has been shown to phosphorylate skeletal-muscle troponin I and troponin T (for a summary see Table 1). If one assumes that it also phosphorylates these proteins from heart, phosphorylase kinase and phospholipid-sensitive Ca^{2+}-dependent protein kinase would be the only two enzymes that can phosphorylate both troponin I and troponin T. Phosphorylase kinase, however, probably is not important in their phosphorylation *in vivo*, because the rate of troponin I phosphorylation by this enzyme is very low compared with the rates seen with phosphorylase or histone (Stull, 1980), and phosphorylation of troponin T was observed even in I strain of mice lacking phosphorylase kinase activity (Moir *et al.*, 1977). It has been suggested that hitherto unidentified protein kinases may be responsible for the phosphorylation of contractile proteins that cannot be solely attributed by phosphorylase kinase or cyclic nucleotide-dependent protein kinases (Moir *et al.*, 1977; Stull, 1980). In the light of its ability to phosphorylate effectively both troponin I and troponin T, as demonstrated in the present studies, the recently recognized phospholipid-sensitive Ca^{2+}-dependent protein kinase (Takai *et al.*, 1979; Kuo *et al.*, 1980) might be one of these enzymes in question.

We have reported that the cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase is localized mostly in the cytosol and, to a smaller extent, in sarcolemmal and microsomal fractions (Katoh & Kuo, 1982). Two of endogenous substrate proteins (M_r 29000 and 38000) for the endogenous enzyme found in heart (Katoh *et al.*, 1981*a*,*b*) have now been tentatively identified, on the basis of their migrations in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, to be troponin I and troponin T (N. Katoh J. F. Kuo, unpublished work). It is noteworthy that Kopp & Barany (1979) have shown that $CaCl_2$ stimulated phosphorylation of troponin I and a 38000-M_r protein resembling troponin T. It is unclear at present, however, whether phosphorylation of troponin I and troponin T by the Ca^{2+}-dependent protein kinase has any

Table 1. *Comparison of phosphorylation of cardiac troponin I and troponin T by various protein kinases*

Data for phospholipid-sensitive Ca^{2+}-dependent protein kinase are taken from the present studies. For determination of K_m and $V_{max.}$, the Ca^{2+}-dependent enzyme ($0.08\,\mu g$) was incubated for 5 min with phosphatidylserine ($5\,\mu g$/0.2 ml) and various concentrations ($0.1–10\,\mu M$) of cardiac troponin I or troponin T, with or without added $CaCl_2$ ($500\,\mu M$); the net activity stimulated by Ca^{2+} was used for the calculation. Data for cyclic AMP-dependent and cyclic GMP-dependent protein kinases are taken collectively from Moir & Perry (1977), Stull & Buss (1977), Blumenthal *et al.* (1978) and Lincoln & Corbin (1978). Data for phosphorylase kinase are taken collectively from Stull *et al.* (1972), Perry & Cole (1974), Rubio *et al.* (1975), Cole & Perry (1975), Moir *et al.* (1977) and Moir & Perry (1980). Data for skeletal-muscle troponin I and troponin T phosphorylation are presented here because of the absence of corresponding studies on cardiac proteins except for cardiac troponin I phosphorylation (Moir & Perry, 1980). Data for troponin T kinase are taken from Villar-Palasi & Kumon (1981). Abbreviations: N.P., not phosphorylated; N.D., not determined.

	Troponin I			Troponin T		
Protein kinase	K_m (μM)	$V_{max.}$ ($\mu mol/min$ per mg)	Phosphorylation (mol of P/mol)	K_m (μM)	$V_{max.}$ ($\mu mol/min$ per mg)	Phosphorylation (mol of P/mol)
Phospholipid-sensitive Ca^{2+}-dependent	3.4 ± 0.9	2.6 ± 0.3	2	0.3 ± 0.1	0.5 ± 0.1	2
Cyclic AMP-dependent	21–26	0.2–11	1–2	N.P.	N.P.	0
Cyclic GMP-dependent	16	0.9	1–2	N.P.	N.P.	0
Phosphorylase kinase	5	N.D.	1	N.D.	N.D.	3
Troponin T kinase	N.P.	N.P.	0	1.5	1.1	1

effects on the Ca^{2+}-sensitivity and/or activity of actomyosin ATPase, similar to certain effects reported by others for the troponin I phosphorylation by other enzymes (Rubio *et al.*, 1975; Solaro *et al.*, 1976; Ray & England, 1976; Reddy & Wyborny, 1976; Bailin, 1979). It appears, however, that phosphorylation of myofibrillar proteins is more probably involved in a long-term modulation of cardiac contractility rather than in the mediation of the acute beat-to-beat function of heart. It is entirely possible that troponin could be phosphorylated *in vivo* by phospholipid-sensitive Ca^{2+}-dependent protein kinase, since most of the enzyme in heart is present in the cytosolic fraction (Katoh & Kuo, 1982). It remains to be seen, however, that phosphorylation by this protein kinase can actually occur *in vivo* under conditions known to cause an increased intracellular Ca^{2+} concentration. Determination of sites of phosphorylation in troponin I and troponin T, in relation to those phosphorylated by other protein kinases, also remains to be accomplished. It should be emphasized that a strict correlation between the data obtained *in vivo* and *in vitro* would be difficult, if not impossible, to obtain unless dephosphorylation of all phosphorylation sites likely to occur during the contractile protein preparation can be uniformly and completely inhibited.

We have observed several endogenous substrate proteins for phospholipid-sensitive Ca^{2+}-dependent protein kinase in guinea-pig hearts (Katoh *et al.*, 1981*a*,*b*). It remains to be determined whether two of these phosphoproteins having apparent molecular weights of 38000 and 29000 are troponin T and troponin I respectively. It may be worth noting here that one of endogenous substrate proteins for phospholipid-sensitive Ca^{2+}-dependent protein kinase found in brain (Wrenn *et al.*, 1980, 1981) has now been positively identified as myelin basic protein (Turner *et al.*, 1982), which is a superior substrate for this enzyme compared with other protein kinases (Wise *et al.*, 1982*b*).

We have shown that certain lipophilic agents, such as adriamycin (Katoh *et al.*, 1981*a*), palmitoylcarnitine (Katoh *et al.*, 1981*b*), trifluoperazine (Wrenn *et al.*, 1981), melittin (Katoh *et al.*, 1982) and compound W-7 (Wise *et al.*, 1982*b*), inhibited phospholipid/Ca^{2+}- or calmodulin/Ca^{2+}-stimulated phosphorylation of various endogenous proteins in tissues (including heart) and inhibited phosphorylation of exogenous proteins (e.g. histone) by phospholipid-sensitive Ca^{2+}-dependent protein kinase. These agents, all having some effects on heart, inhibited the ability of this enzyme to phosphorylate cardiac troponin I and troponin T (Table 2). Trifluoperazine (Weiss & Wallace, 1980) and compound W-7 (Hidaka *et al.*, 1979) have been shown to inhibit various calmodulin/Ca^{2+}-stimulated enzymes and have often been regarded as 'selective' calmodulin antagonists. Verapamil, felodipine, diltiazem, nifedipine and prenylamide, hydrophobic agents shown to block the slow Ca^{2+} channel (for a recent monograph see Weiss, 1981), on the other hand, were without effect (Table 2). It appears that the present enzyme probably is not a potential site of direct actions of these cardioactive drugs *in vivo*, and that the enzyme may not be involved in Ca^{2+} transport through the membrane via this channel. Forskolin, a cardioactive diterpene (Lindner *et al.*, 1978), has been shown to be able to stimulate

Table 2. *Comparative effects of various agents on the phosphorylation of cardiac troponin I and troponin T by cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase*

Phosphorylation of troponin I (10 μg) and troponin T (5 μg) by the enzyme (0.08 μg) was performed with or without $CaCl_2$ (500 μM), in the presence of phosphatidylserine (5 μg/0.2 ml) and various agents, as indicated. The values presented are the means of duplicated incubations, with assay errors being less than 5%.

	Protein kinase activity (pmol of P incorporated/min)			
	Troponin I		Troponin T	
Addition	Basal	$+Ca^{2+}$	Basal	$+Ca^{2+}$
None (control)	1.2	18.5	2.5	20.5
Adriamycin (100 μM)	1.1	7.4	2.5	11.5
Palmitoylcarnitine (30 μM)	1.2	2.0	2.3	3.2
Trifluoperazine (40 μM)	1.0	3.7	1.9	5.0
Melittin (4 μM)	1.2	7.6	2.5	7.3
Compound W-7 (100 μM)	1.1	9.4	2.4	9.9
Ca^{2+} antagonists* (10–200 μM)	0.9–1.2	16.8–19.2	2.0–2.5	19.5–21.0
Forskolin (10–240 μM)†	1.3–1.4	16.2–20.2	2.1–2.6	18.2–23.6
Ouabain (10–1000 μM)	1.2–1.5	17.5–20.8	2.4–2.8	19.8–22.2

* Including verapamil, felodipine, diltiazem, nifedipine and prenylamine; dissolved in water or ethanol.
† Dissolved in ethanol or dimethyl sulphoxide.

adenylate cyclase directly (Seaman & Daly, 1981) and to augment receptor-mediated activation of the cyclic AMP-generating system in membrane (Daly *et al.*, 1982). We have noted that forskolin was without effect on the present enzyme (Table 2). Ouabain, a cardiac glycoside, was also inactive (Table 2). Inhibition and lack of inhibition by these agents, mentioned in Table 2, of troponin I and troponin T phosphorylation by the enzyme were also observed when histone Hl was used as substrate (Wise *et al.*, 1982*b*; N. Katoh, B. C. Wise & J. F. Kuo, unpublished work).

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