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Phosphorylation of Cardiac Troponin by Guanosine 3′:5′-Monophosphate-dependent Protein Kinase*

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SUMMARY

Homogeneous cGMP-dependent protein kinase catalyzes the rapid incorporation of phosphate, specifically into the inhibitory subunit of purified cardiac troponin with a maximal incorporation of 1 mol of phosphate/mol of troponin. When troponin was incubated in the presence of both cGMP- and cAMP-dependent protein kinases, a maximal incorporation of 1 mol of phosphate/mol of troponin was observed which suggested phosphorylation of the same site by the two kinases. Both cyclic nucleotide-dependent kinases had similar K_m values for troponin, but the V_{max} value for the phosphorylation reaction catalyzed by cAMP-dependent protein kinase was 12-fold greater than the value obtained for cGMP-dependent protein kinase.

Cyclic AMP- and cGMP-dependent protein kinases have recently been obtained in homogeneous form and have been observed to differ in cyclic nucleotide specificity and subunit composition (1–7). Although a number of substrates for cAMP-dependent protein kinase have been identified, little is known concerning physiologically relevant substrates for cGMP-dependent protein kinase. Because differing physiological effects are associated with elevated cellular concentrations of cAMP and cGMP, Goldberg and co-workers (8) have proposed that the two cyclic nucleotides mediate different and often opposing biochemical processes. This would imply phosphorylation of different substrates by the two cyclic nucleotide-dependent protein kinases. Although initial studies indicated that partially purified cGMP-dependent protein kinase was unable to activate phosphorylase kinase or inactivate glycogen synthase (9–11), two well documented substrates for cAMP-dependent protein kinase (12), recent studies have demonstrated that purified cGMP-dependent protein kinase catalyzed similar phosphorylation and/or activation of hormone-sensitive lipase, phosphorylase kinase, glycogen synthase, pyruvate kinase, fructose-1,6-diphosphatase, and histone (7, 13, 14). Troponin, the Ca^{2+} regulatory protein of cardiac myofibrils, is another well documented substrate for cAMP-dependent protein kinase. Phosphorylation of the troponin subunit which inhibits actomyosin ATPase activity (TN-I)[1] occurs in the intact rat heart when cAMP concentrations are elevated due to β-adrenergic stimulation (15) and *in vitro* when incubated in the presence of cAMP-dependent protein kinase (16, 17). In contrast to the increases in cardiac cAMP concentrations and contractile force observed with β-adrenergic agents, cholinergic stimulation has been shown to cause elevations in cGMP content (15, 18, 19), depression of the contractile state of the heart (18), and an attenuation of the positive inotropic effects of isoproterenol and histamine (19). These studies indicate that changes in the contractile state of the heart associated with elevations in cAMP and cGMP content differ and may be related to the phosphorylation of cardiac troponin. This and the accompanying paper (20) report that cGMP-dependent protein kinase catalyzes the rapid phosphorylation of troponin. We have examined the characteristics of the phosphorylation catalyzed by cGMP-dependent protein kinase in order to compare them with those obtained for cAMP-dependent protein kinase.

EXPERIMENTAL PROCEDURES

Materials—Histone H_{2b}, buffers, and cyclic nucleotides were obtained from Sigma Chemical Co. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (21).

Preparation of Proteins—Bovine cardiac troponin·tropomyosin complex (TN·TM) and troponin (TN) were purified by the method of Stull and Buss (17). TN·TM and TN have apparent molecular weights of 150,000 and 80,000, respectively. The purified proteins were free of detectable kinase or phosphatase activity.

Cyclic GMP-dependent protein kinase was purified from bovine lung by affinity chromatography on $8NH_2(CH)_2NH$/cAMP/Sepharose (3, 5). The homogeneous enzyme had a specific activity of 1375 units/mg. One unit of activity is that amount of enzyme which catalyzes the transfer of 1 nmol of ^{32}P from [γ-^{32}P]ATP into histone H_{2b} per min at pH 6.8 and 30°.

Heat-stable protein inhibitor of cAMP-dependent protein kinase was prepared by the method of Ashby and Walsh (22).

Enzyme Assay—Reaction mixtures containing 50 mM Tes, pH 8.0, 4 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 0.4 μM cGMP or 10 μM cAMP, 0.25 mM [γ-^{32}P]ATP (200 cpm/pmol), and TN·TM or TN were incubated with cGMP- or cAMP-dependent protein kinase at 30°. Aliquots (20 μl) were removed, spotted on Whatman No. 3MM filter paper squares, and processed as described (17) for measurements of protein-bound phosphate.

RESULTS

Phosphorylation of Troponin·Tropomyosin and Troponin by cGMP-dependent Protein Kinase—Incubation of bovine cardiac TN·TM complex with cGMP-dependent protein kinase resulted in the rapid incorporation of ^{32}P into protein (Fig. 1). No ^{32}P incorporation was noted with TN·TM or cGMP-dependent protein kinase alone. Phosphorylation was largely complete by 40 min with a maximal incorporation of 0.82 ± 0.06 mol of ^{32}P incorporated/mol of TN·TM. A similar extent of phosphorylation was observed with TN in the absence of TM (1.02 ± 0.03 mol of ^{32}P/mol of TN). The rate of phosphorylation

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[1] The abbreviations used are: TN-I, TN-C, and TN-T designate the inhibitory, calcium-binding, and tropomyosin-binding subunits of troponin, respectively; TM, tropomyosin; TN, troponin; TN·TM, troponin·tropomyosin; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

of troponin by cGMP-dependent protein kinase was stimulated 4-fold in the presence of 0.5 μM cGMP. The concentrations of cGMP and cAMP required for half-maximal stimulation with troponin as substrate were approximately 0.1 and 4 μM, respectively. The addition of the heat-stable protein inhibitor of cAMP-dependent protein kinase in quantities which inhibited the rate of phosphorylation of TN catalyzed by cAMP-dependent protein kinase by more than 80% had no effect on the rate or extent of phosphorylation of TN catalyzed by cGMP-dependent protein kinase. These results concerning the effects of cGMP, cAMP, and the heat-stable protein kinase inhibitor are in agreement with previous reports which used histone as a substrate for the cGMP-dependent protein kinase (3, 4, 23).

In order to determine whether the value of ^{32}P incorporation into troponin after prolonged incubation reflected a saturation of phosphorylatable sites, additional troponin was added to the reaction mixture after 60 min of incubation. The rate and extent of phosphorylation after addition of troponin was identical with that observed during the first 60 min of incubation, indicating no significant loss of cGMP-dependent protein kinase activity or [γ-^{32}P]ATP.

Identification of the Phosphorylated Subunit—The stoichiometry of 1 mol of ^{32}P incorporated/mol of TN suggested phosphorylation of a single site by cGMP-dependent protein kinase, analogous to the results obtained previously with cAMP-dependent protein kinase (16, 17). Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that 88% of the radioactivity applied to the gel was associated with the TN-I subunit (Fig. 2). Identical results were obtained with bovine cardiac TN·TM. Because catalytic amounts of enzyme were used, autophosphorylation of cGMP-dependent protein kinase was not observed (24).

Comparison of Phosphorylation of Troponin by cGMP- and cAMP-dependent Protein Kinases—In order to determine whether cGMP- and cAMP-dependent protein kinases catalyzed phosphorylation of the same or different sites in TN-I, the effect of sequential additions of the two enzymes on the extent of phosphorylation was examined. As shown in Fig. 3, the maximal extent of phosphorylation catalyzed by each enzyme alone was similar (cAMP-dependent protein kinase, 1.10 ± 0.03 (n = 3) *versus* cGMP-dependent protein kinase, 1.02 ± 0.03 (n = 4) mol of ^{32}P incorporated/mol of TN). When cAMP-dependent protein kinase was added to the reaction mixture containing TN which had been partially phosphorylated by cGMP-dependent protein kinase (0.44 mol of ^{32}P incorporated/mol of TN), the maximal extent of phosphorylation observed in the presence of both enzymes (1.01 ± 0.01 (n = 3) mol of ^{32}P incorporated/mol of TN) was not significantly different from that catalyzed by either enzyme alone. A value of 2 mol of ^{32}P incorporated/mol of TN would be expected if separate sites were phosphorylated by the respective kinases.

Initial rates of TN phosphorylation were examined in order to compare kinetic parameters of the reactions catalyzed by the two kinases. As shown in Table I, the K_m and V_{max} values

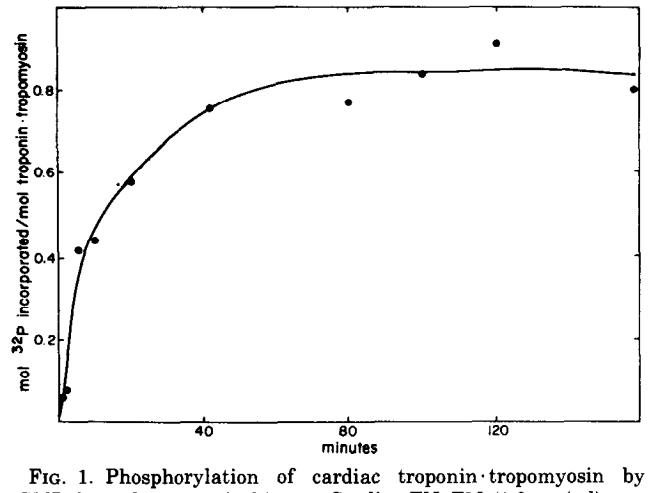

FIG. 1. Phosphorylation of cardiac troponin·tropomyosin by cGMP-dependent protein kinase. Cardiac TN·TM (1.2 mg/ml) was incubated as described under "Experimental Procedures" with cGMP-dependent protein kinase (0.58 units/ml).

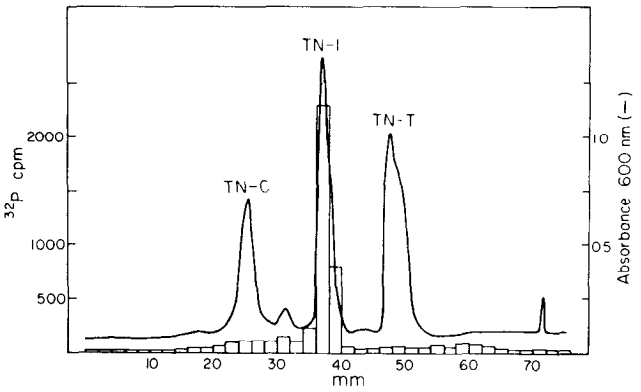

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cardiac troponin phosphorylated by cGMP-dependent protein kinase. Cardiac TN phosphorylated as described under "Experimental Procedures" was precipitated with 15% trichloroacetic acid and then dissolved in 1% sodium dodecyl sulfate. Polyacrylamide gel electrophoresis was performed as previously described (17). Destained gels were scanned for Coomassie blue absorbance at 600 nm (*solid line*) and then sequentially sliced for quantitation of ^{32}P incorporation (*bars*).

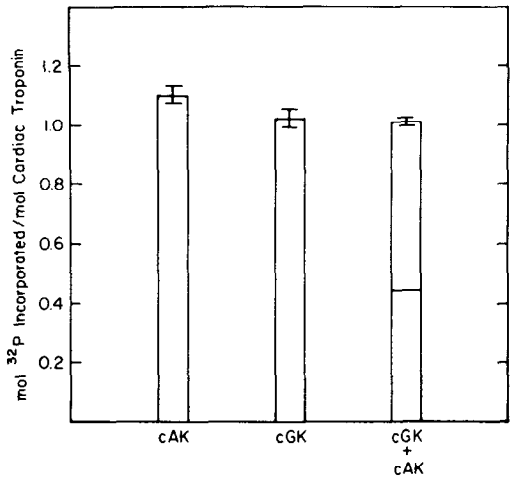

FIG. 3. Phosphorylation of cardiac troponin by cAMP- and cGMP-dependent protein kinases. Reaction mixtures were incubated as described under "Experimental Procedures" with the following additions: *cAK*, TN (0.39 mg/ml) and cAMP-dependent protein kinase (1.31 units/ml); *cGK*, TN (0.70 mg/ml) and cGMP-dependent protein kinase (13.2 units/ml). For *cGK + cAK*, cardiac TN (0.39 mg/ml) and cGMP-dependent protein kinase (0.01 units/ml) were incubated until 0.44 mol of ^{32}P was incorporated/mol of TN. Cyclic AMP-dependent protein kinase (1.08 units/ml) and cAMP (10 μM) were then added to the reaction mixture and incubation continued until maximal phosphorylation had occurred. The maximal extent of phosphorylation indicated is the mean ± S.E. for at least three values.

TABLE I

Kinetic parameters for phosphorylation of cardiac troponin by cGMP-dependent and cAMP-dependent protein kinases

Kinase	K_m	V_{max}
	μM	$\mu mol\ ^{32}P/min/mg\ kinase^a$
cGMP-dependent	16.1 ± 3.6	0.93 ± 0.27
cAMP-dependent[b]	20.8 ± 2.5	10.9 ± 1.3

[a] V_{max} values are based upon the phosphorylation catalyzed by homogeneous preparations of the two kinases. One milligram of homogeneous cGMP-dependent protein kinase catalyzed the incorporation of 1375 nmol of ^{32}P/min into histone H_{2b} at pH 6.8 (3), while 1.0 mg of homogeneous cAMP-dependent protein kinase catalytic subunit catalyzed the incorporation of 3300 nmol of ^{32}P/min into histone type IIA at pH 6.5 (2).

[b] Homogeneous catalytic subunit of cAMP-dependent protein kinase was used to determine the K_m and V_{max} values presented. The V_{max} value has been corrected to reflect the specific activity of homogeneous holoenzyme for purposes of comparison.

for the reaction catalyzed by cGMP-dependent protein kinase were found to be 16.1 ± 3.6 μM and 0.93 ± 0.27 μmol of ^{32}P incorporated/min/mg of purified kinase, respectively. The K_m and V_{max} values obtained for cAMP-dependent protein kinase were 20.8 ± 2.5 μM and 10.9 ± 1.3 μmol of ^{32}P incorporated/min/mg of purified holoenzyme, respectively. The maximal rate of phosphorylation of TN catalyzed by cGMP-dependent protein kinase occurred between pH 8.0 and 9.0, similar to that previously reported for the cAMP-dependent protein kinase-catalyzed reaction (17).

DISCUSSION

The present studies indicate that cGMP-dependent protein kinase catalyzes the maximal incorporation of 1 mol of ^{32}P/mol of TN-I, identical with that previously reported for cAMP-dependent protein kinase (17). Incubation of TN in the presence of both kinases also results in a maximal extent of phosphorylation of 1 mol of ^{32}P/mol of TN, suggesting that both kinases catalyze phosphorylation of the same site in TN-I. Because studies in the intact heart indicate that phosphorylation of TN-I occurs in response to elevated concentrations of cAMP, but not to elevated concentrations of cGMP (15), the kinetics of the two *in vitro* reactions were compared. Although the K_m values for troponin phosphorylation by cAMP- and cGMP-dependent protein kinase were similar, the V_{max} values for the cAMP-dependent protein kinase-catalyzed reaction exceeded that for the cGMP-dependent protein kinase-catalyzed reaction by 12-fold. Taken in conjunction with the apparent 11-fold greater amounts of cAMP-dependent protein kinase present in the heart (25), phosphorylation of TN-I by cAMP-dependent protein kinase in the intact heart would occur at a rate 130-fold greater than the cGMP-dependent reaction, if both kinases were fully activated. The kinetic studies presented here are the first which can satisfactorily explain why elevations in cGMP content in the intact heart are not associated with TN-I phosphorylation (15). Increases in cGMP concentration in the intact heart are instead associated with the dephosphorylation of TN-I (15), suggesting a complex role for cGMP in regulating the phosphorylation and dephosphorylation of TN-I.

Recently a comparison of cGMP-dependent protein kinase with cAMP-dependent protein kinase suggested structural and evolutionary parallelisms in the two enzymes (5–7). It is, therefore, not surprising to find that the two enzymes are capable of phosphorylating some of the same substrates *in vitro*, albeit at different rates. However, cAMP and cGMP often appear to mediate differing and opposing physiological effects, presumably by stimulating phosphorylation of different substrates by the respective kinases. Thus, many substrates which may be phosphorylated *in vitro* by both kinases are apparently only phosphorylated *in vivo* by one of the kinases at a rate sufficient to result in a biochemical effect. The results presented here emphasize the importance of obtaining kinetic parameters for the phosphorylation of proteins *in vitro* in order to evaluate the potential significance of such reactions *in vivo*.

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