Phosphorylation of Cardiac Troponin by Cyclic Adenosine 3':5'-Monophosphate-dependent Protein Kinase*

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The purpose of this investigation was to characterize the phosphorylation of bovine cardiac troponin by cyclic AMP-dependent protein kinase. The purified troponin-tropomyosin complex from beef heart contained 0.78 ± 0.13 mol of phosphate per mol of protein. Analysis of the isolated protein components indicated that the endogenous phosphate was predominately in the inhibitory subunit (TN-I) and the tropomyosin-binding subunit (TN-T) of troponin. When cardiac troponin or the troponin-tropomyosin complex was incubated with cyclic AMP-dependent protein kinase and [γ-32P]ATP, the rate of phosphorylation was stimulated by cyclic AMP and inhibited by the heat-stable protein inhibitor of cyclic AMP-dependent protein kinase. The 32P was incorporated specifically into the TN-I subunit with a maximal incorporation of 1 mol of phosphate per mol of protein. The maximal amount of incorporated phosphate did not vary significantly between troponin preparations that contained low or high amounts of endogenous phosphate. The Vmax of the initial rates of phosphorylation with troponin or troponin-tropomyosin as substrates was 3.5-fold greater than the value obtained with unfractonated histones. The rate or extent of phosphorylation was not altered by actin in the presence or absence of Ca2+. The maximal rate of phosphorylation occurred between pH 8.5 and 9.0. At pH 6.0 and 7.0 the maximal rates of phosphorylation were 13 and 45% of that observed at pH 8.5, respectively. These results indicate that cyclic AMP formation in cardiac muscle may be associated with the rapid and specific phosphorylation of the TN-I subunit of troponin. The presence of endogenous phosphate in TN-T and TN-I suggests that kinases other than cyclic AMP-dependent protein kinase may also phosphorylate troponin in vivo.

Recently, investigations in several different laboratories have indicated a possible role for the phosphorylation of the Ca2+ regulatory protein of cardiac myofibrils, troponin, in mediating the positive inotropic effects associated with cyclic AMP formation. Cardiac troponin is composed of three distinct subunits which are functionally related to the three distinct subunits of skeletal muscle troponin (1): TN-I, subunit which binds calcium; TN-T subunit which binds tightly to tropomyosin. In the presence of all three subunits, actomyosin ATPase activity is inhibited in the absence of Ca2+ and stimulated in the presence of Ca2+. Reddy et al. (2) reported that purified cardiac troponin-tropomyosin contained a small amount of kinase activity which was stimulated almost 2-fold by cyclic AMP. Recently it has been shown that purified cardiac TN-I as well as TN-I in the troponin complex were phosphorylated by cyclic AMP-dependent protein kinase (3). A small amount of phosphate was also incorporated into TN-T. The phosphorylation of purified cardiac TN-I was not inhibited by the addition of TN-C whereas skeletal muscle TN-C inhibited the phosphorylation of skeletal muscle TN-I. Epinephrine administration to perfused rat hearts resulted in the phosphorylation of TN-I which was closely correlated with the enhancement of contractile activity (4). Isoproterenol did not stimulate the phosphorylation of skeletal muscle troponin in vivo (5). These results emphasize the differences in the troponin subunits from heart and skeletal muscle. It has also been reported that the phosphorylation of cardiac troponin by cyclic AMP-dependent protein kinase slightly increased the calcium sensitivity of the actomyosin ATPase activity (6). The increased Ca2+ sensitivity may be mediated by changes in Ca2+ binding to troponin, but other possible mechanisms cannot be excluded at this time. These reports strongly imply that the positive inotropic effects of catecholamines on cardiac muscle may be mediated by the phosphorylation of the troponin subunits.

Before the biochemical effect of phosphorylation on troponin activity can be studied in detail, it is necessary to have a complete description of the phosphorylation reaction. Measurements of the amount of phosphate incorporated into TN-I previously indicated a variable incorporation of 0.1 to 1.0 mol of phosphate per mol of TN-I (2, 3). Also phosphate incorporated into cardiac troponin may be removed by phosphatase activity contaminating the reaction mixture (3). Thus, a variable amount of incorporated phosphate which may be readily lost presents serious difficulties in attempts to define the biochemical significance of the phosphorylation reaction in relation to the function of troponin in myofibrils. Additionally, cyclic AMP-dependent protein kinase is clearly recognized as an enzyme capable of catalyzing the non-specific incorporation

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1 The abbreviations used are: TN-I, TN-T, and TN-C are used to designate the inhibitory, tropomyosin-binding, and calcium-binding subunits of troponin, respectively; Mops, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate.

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of $^{32}$P into many proteins not normally phosphorylated (7). It is important to evaluate the phosphorylation reaction found with purified troponin to determine if the essential characteristics are consistent with the observations made on the phosphorylation of cardiac troponin in intact tissue. Therefore, the purpose of this investigation was to examine the characteristics of the phosphorylation of bovine cardiac troponin catalyzed by cyclic AMP-dependent protein kinase and to determine the effects of the other proteins of the thin filament, actin and tropomyosin, on the phosphorylation reaction. A method for the purification of the contractile proteins was used to eliminate significant amounts of contaminating kinase, phosphatase, and protease activities for these studies.

EXPERIMENTAL PROCEDURES

Materials – CM-agarose, Bio-Gel A-0.5m, and all electrophoretic chemicals were obtained from Bio-Rad Laboratories. Biochemicals, buffers, and unfractionated histones (type II-A) were purchased from Sigma Chemical Co. The 2-mercaptoethanol was distilled before use. Triton X-100 was obtained from Rohm and Haas, Co., fluorescamine from Hoffman-La Roche, and Omnifluor from New England Nuclear.

Preparation of Proteins – All procedures were performed at 0-4°C unless noted otherwise. Ventricular muscle from beef hearts was homogenized in 7 volumes of 50 mM KCl, 20 mM Tris, 2 mM EDTA, and 15 mM 2-mercaptoethanol at pH 8.0. The myofilibrils were collected at 5000 $x g$ for 10 min and were centrifuged in the same buffer containing 1% Triton X-100. The precipitate was then collected by centrifugation repeated three times in the buffer solution without the Triton X-100.

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Protein Measurement – Fluorescamine was used to measure protein concentrations with bovine serum albumin as a standard (15). The fluorescence assay of the purified cardiac proteins was standardized by the biuret reaction for protein.

RESULTS

Protein Purification – Purification of beef cardiac troponin by the described procedure produces a highly purified protein complex of the three distinct subunits of troponin and tropomyosin (Fig. 1, Gel a). In contrast to the purification procedure for skeletal muscle troponin - tropomyosin, it was necessary to include a procedure for solubilizing membrane material with Triton X-100 during the initial steps of the purification (16). The Triton X-100 improved the degree of purification and also appeared to eliminate significant contamination with myocardial proteolytic enzymes in that the purified proteins were not converted to smaller molecular weight components during storage for several days at 0°C or during incubation at 30°C for several hours. This was an important feature of the purification procedure since it would be unacceptable to have protein substrates hydrolyzed under the conditions of the phosphorylation experiments. A protein with an apparent molecular weight of approximately 24,000 was found in small amounts as a frequent contaminant. The inclusion of the gel filtration procedure separated this minor component from the troponin - tropomyosin complex. These results suggest that the protein was a separate entity which did not bind to any of the protein components of the troponin - tropomyosin. This obser-
who suggested the 24,000-dalton component was an electro-
phoretic artifact related to an intramolecular disulfide link of
TN-I. The coincident migration of the troponin subunits and
tropomyosin was separated from TN-C by adjusting the pH to
4.6 in the presence of 1 M KC1 and collecting the precipitated
protein. The pH of both the supernatant solution and pellet was
adjusted to pH 7.0 with 1 N NaOH. The solutions were
dialyzed extensively against 10 mM Mops and 15 mM 2-mercapto-
ethanol at pH 7.0. Tropomyosin was precipitated by adjusting the pH to 4.6 and was
collected by centrifugation. The pH of the supernatant solution
and pellet was adjusted to pH 7.0 with 1 N NaOH. The solutions were
dialyzed extensively against 10 mM Mops and 15 mM 2-mercapto-
ethanol at pH 7.0. The fractions between 280 to 320 ml (Fraction 2) and
390 to 415 ml (Fraction 3) were pooled and dialyzed extensively
against 0.01 N HCl and 15 mM 2-mercaptoethanol. The recovery of
protein was 90%.

**TABLE I**

**Protein-bound phosphate in troponin subunits and tropomyosin**

| Protein fraction | Phosphate content | Proteinbound phosphatecontent | Proteinbound phosphatecontent | Proteinbound phosphatecontent |
|------------------|-------------------|-------------------------------|-------------------------------|-------------------------------|
| Troponin · tropomyosin | 0.56 ± 0.10 | 0.78 ± 0.15 | Tropomyosin plus TN-C | 0.19 ± 0.09 | 0.67 ± 0.01 |
| TN-T             | 0.51 ± 0.08 | 0.21 ± 0.03 | TN-I              | 1.22 ± 0.27 | 0.34 ± 0.09 |

* The apparent molecular weights used were: tropomyosin, 35,000 (monomer); TN-C, 18,500; TN-I, 28,000; TN-T, 40,000.

**Phosphorylation by cyclic AMP-dependent protein kinase**

Incubation of the purified troponin · tropomyosin complex
with cyclic AMP-dependent protein kinase in the presence of
cyclic AMP, [γ-32P]ATP, and Mg2+ led to a rapid incorporation
of 32P into protein (Fig. 3). The maximal amount of 32P incorporated
was 1.20 ± 0.06 mol of phosphate per mol of troponin · tropomyosin. There was no significant difference in the maximal
amount of phosphate incorporated in cardiac troponin · tropomyosin preparation that contained little endogenous
phosphate (0.30 mol of phosphate per mol of protein) and those preparations with high endogenous phosphate (0.98 mol of phosphate per mol of protein). No 32P was incorporated into troponin · tropomyosin unless cyclic AMP-dependent kinase
was added. These results indicated that the cardiac tropo-
nin - tropomyosin fraction was not contaminated with kinase activity.

The phosphorylated cardiac troponin - tropomyosin was precipitated with ammonium sulfate adjusted to 60% saturation and dissolved in 0.2 M KCl, 10 mM Mops, pH 7, and 15 mM 2-mercaptoethanol. After dialysis overnight against the same solution there was no loss of the incorporated 32P. Also, incubation of the phosphorylated protein in the presence of 2 mM MgCl₂ for 4 hr at 30° did not result in any measurable loss of protein-bound phosphate or any significant change in the protein pattern after polyacrylamide gel electrophoresis in the presence of SDS. These results demonstrated the absence of significant phosphatase or protease activity in the cardiac troponin - tropomyosin that would hydrolyze phosphoester or peptide bonds, respectively.

The addition of the heat-stable inhibitor protein of cyclic AMP-dependent protein kinase decreased the rate of phosphorylation of cardiac regulatory proteins (Fig. 4). In the presence of 10 μM cyclic AMP and added cyclic AMP-dependent protein kinase the reaction rate was inhibited in proportion to the amount of inhibitor protein added with an inhibition of 91% in the presence of 10 μg of inhibitor protein. In the absence of cyclic AMP, the rate of phosphorylation was only 15% of that observed in the presence of the nucleotide. The inhibition of this slower rate of phosphorylation by the heat-stable inhibitor protein suggested that this reaction was also catalyzed by the cyclic AMP-dependent protein kinase (17). The concentration of cyclic AMP required for stimulation to half of the maximal rate was approximately 0.1 μM (data not shown).

Identification of Phosphorylated Subunits – The stoichiometric amount of phosphate incorporated into the purified troponin - tropomyosin suggested that only one of the protein subunits might be specifically phosphorylated. Analysis of the 32P incorporation into the protein by polyacrylamide gel electrophoresis in the presence of SDS indicated the phosphate was incorporated into the TN-I subunit by the cyclic AMP-dependent protein kinase (Fig. 5). A small amount of radioactivity (usually between 4 and 6%) was not associated with TN-I. This radioactivity was near TN-C, but the diffuseness of the pattern did not indicate a specific association with TN-C or any other protein component. The nature of this radioactivity was not further investigated. The phosphorylation of TN-I was also observed when troponin was phosphorylated in the absence of tropomyosin. Although TN-T from cardiac muscle contained protein-bound phosphate it was not phosphorylated by cyclic AMP-dependent protein kinase in these experiments either in the presence or absence of tropomyosin.

Effect of pH on Phosphorylation of Troponin – The effect of pH on the rate of phosphorylation of cardiac troponin was determined (Fig. 6). The optimal pH for the cyclic AMP-dependent protein kinase-catalyzed reaction was between pH 8.5 and 9.0, which is much greater than the observed pH optimum for other protein substrates such as casein or histones. This effect of pH was found both with cardiac troponin or troponin - tropomyosin complex. The rates of phosphorylation at pH 6.0 and 7.0 in eight experiments were 12.8 ± 1.3% and 45 ± 3.8%, respectively, of the rate obtained at pH 8.5. The phosphorylation at pH 6.0 was also associated with 32P incorporation into the TN-I component. Also, the maximal amount of phosphate incorporated at pH 6.0 was similar to the amount incorporated at pH 8.5. The effect of pH on phosphorylation of cardiac troponin may explain the slow rate of phosphorylation observed by Reddy et al. (2) who used pH 6.2 for their incubation conditions.

Effect of Other Proteins on Phosphorylation Reaction – A comparison was made between the kinetic parameters of the initial rates of phosphorylation of cardiac troponin and several other protein substrates of cyclic AMP-dependent protein kinase under conditions which were optimal for each substrate (Table II). A comparison with the values obtained with unfractionated histones, a commonly used protein substrate in vitro, indicated that the cardiac proteins had Vₘₐₓ values approximately 3.5 times the Vₘₐₓ value of the histones. The removal of tropomyosin from cardiac troponin did not significantly alter the kinetic values of the phosphorylation reaction. At pH 6.0, the Kₘ value for the phosphorylation of cardiac troponin - tropomyosin was decreased to 17 ± 11 μM. However, the slower rate of phosphorylation at the lower pH could be explained by the marked decrease in the Vₘₐₓ value to 31 ± 18 nmol of 32P/min/mg of enzyme. The Vₘₐₓ value of skeletal muscle troponin was only 2% of the histone value which indicated that skeletal muscle troponin was not a good substrate for the kinase.²

² J. T. Stull and J. E. Buss, unpublished observations.
Phosphorylation of Cardiac Troponin

Fig. 5. Polyacrylamide gel electrophoresis of phosphorylated cardiac troponin-tropomyosin (A) and troponin (B). Cardiac troponin-tropomyosin or troponin was incubated for 50 min. with cyclic AMP-dependent protein kinase as described in Fig. 3. The protein was precipitated with 200 μl of 5% trichloroacetic acid and 0.25% sodium tungstate, pH 1.8. The protein was collected by centrifugation and prepared for polyacrylamide gel electrophoresis in the presence of SDS. The destained gels were scanned for Coomassie blue absorbance at 600 nm and then sliced sequentially for measurement of 32P.

Fig. 6. Effect of pH on the phosphorylation of cardiac troponin-tropomyosin by cyclic AMP-dependent protein kinase. Cardiac troponin-tropomyosin (1.7 mg/ml) was incubated with 8 μg/ml of cyclic AMP-dependent protein kinase under standard reaction conditions with the exception that the reactions contained 50 mM buffer at the indicated pH values. The buffers were 2-(N-morpholino)ethanesulfonic acid (●), N-[Tris(hydroxymethyl)methyl-2-aminol octanesulfonic acid (△), or Tris (○).

Table II

| Protein                  | K<sub>v</sub> (μM) | V<sub>max</sub> (μmol 32P/min/mg protein) |
|--------------------------|------------------|----------------------------------------|
| Unfractionated histones  | 30 ± 1.8<sup>a</sup> | 80 ± 3.3                                |
| Cardiac troponin·tropomyosin | 56 ± 19            | 214 ± 73                                |
| Cardiac troponin         | 57 ± 14           | 341 ± 36                                |

<sup>a</sup> Average molecular weight assumed to be 16,000.

Discussion

Beef cardiac troponin is a phosphoprotein of the myofibrils whose function may be regulated by phosphorylation and dephosphorylation reactions. Although skeletal muscle troponin also contains protein-bound phosphate (18), differences between the cardiac and skeletal muscle proteins suggest different roles for the phosphorylation. The subunit of skeletal muscle troponin which contains most of the phosphate was identified as TN-T (19, 20). This is in contrast to results reported elsewhere observations by others (3) where the TN-I subunit from cardiac muscle contains most of the phosphate. A significant portion of the phosphate in cardiac troponin was found in TN-T as well. Skeletal muscle troponin is not phosphorylated in response to tetanic contractions (5, 21) or after...
Phosphorylation of Cardiac Troponin

![Phosphorylation of Cardiac Troponin](https://example.com/fig7.png)

**FIG. 7.** Effect of actin and Ca$^{2+}$ on the phosphorylation of cardiac troponin. General reaction conditions were the same as described under "Experimental Procedures" with 1.1 mg/ml of cardiac troponin-tropomyosin, 82 μg/ml of cyclic AMP-dependent protein kinase in the absence (○) or presence (△) of 2.1 mg/ml of polymerized actin. Also included was 10 μM CaCl$_2$ (○, △) and 0.5 mM ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (●, ▲).

The stimulation of cyclic AMP formation by isoproterenol in *vivo* (5). These results have prevented the description of a physiological role for a phosphorylation reaction involving skeletal muscle troponin. However, heart troponin was rapidly phosphorylated in response to epinephrine (4) in a manner that suggested an important role in regulation of contractile activity. The amount of endogenous phosphate measured in the purified cardiac troponin would depend upon the relative kinase and phosphatase activities present during the time of purification. Since ATP was not added to the solutions used in the purification procedure, phosphatase activity would be relatively greater than kinase activity. Thus the amounts of endogenous phosphate found in the troponin-tropomyosin fraction and in the purified subunits are likely to reflect values lower than the maximal amount which could be obtained in *vivo*. The phosphatase and kinase enzymes are probably destroyed by the exposure to ethanol and diethyl ether.

In order to investigate the phosphorylation of cardiac troponin by cyclic AMP-dependent protein kinase, it was necessary to establish that the reaction was not catalyzed by multiple kinases either in the troponin-tropomyosin fraction or from the purified cyclic AMP-dependent protein kinase solution (17). This is a rather important point in interpreting data related to the specificity of various protein kinases. For example, it was suggested that phosphorylase kinase catalyzed the phosphorylation of a light chain of myosin from rabbit skeletal muscle (22). Later investigations showed the reaction was catalyzed by a different enzyme from skeletal muscle, a myosin light chain kinase (23). Since no phosphorylation of cardiac troponin was observed in the present investigation unless cyclic AMP-dependent protein kinase was added to the reaction mixture, the cardiac troponin-tropomyosin preparation was not contaminated with significant kinase activity. This is in contrast to previous reports (2, 3). It has been shown in this report that troponin phosphorylation was stimulated by cyclic AMP with added cyclic AMP-dependent protein kinase. The heat-stable inhibitor protein markedly decreased the rate of phosphorylation stimulated by cyclic AMP as well as the slower reaction observed in the absence of this nucleotide. This would indicate that the slower reaction was catalyzed by some dissociated catalytic subunit of the cyclic AMP-dependent protein kinase since the inhibitor protein specifically binds to the dissociated catalytic subunit (17). Up to 84 and 94% inhibition of the rate of the phosphorylation reactions were observed in the absence and presence of cyclic AMP, respectively. Therefore it is reasonable to assume that the phosphorylation reaction was specifically catalyzed by cyclic AMP-dependent protein kinase. The fact that at least 30% of the phosphorylation of cardiac troponin observed by Cole and Perry (3) was insensitive to the inhibitor protein suggests that there may be kinase activity in cardiac muscle not identical to the cyclic AMP-dependent enzyme.

Cole and Perry also observed significant $^{32}$P incorporation into the TN-T subunit of troponin (3). Although our observations confirm their report of endogenous phosphate in TN-T, we were not able to demonstrate any $^{32}$P incorporation into TN-I in the troponin-tropomyosin complex or troponin alone in the presence of cyclic AMP-dependent protein kinase. We have also found endogenous phosphate in TN-I (0.34 mol/mol of protein), but the amount is substantially less than the value of 1.9 mol/mol of protein reported by Cole and Perry (5). They also found that the amount of phosphate incorporated upon incubation with cyclic AMP-dependent protein kinase varied inversely with the phosphate content of TN-I. Therefore the site phosphorylated by cyclic AMP-dependent protein kinase was probably partially phosphorylated in the purified protein. In our troponin preparations in which there was a significant difference in phosphate content (0.30 to 0.98 mol of phosphate per mol of protein) the maximal amount of phosphate incorporated by cyclic AMP-dependent protein kinase into TN-I was the same. These results suggest that the site phosphorylated by cyclic AMP-dependent protein kinase is completely dephosphorylated with our purification procedure and that another kinase may catalyze the phosphorylation of TN-I in cardiac muscle *in vivo*. These suggestions of kinase activity towards cardiac troponin which is distinct from cyclic AMP-dependent protein kinase demonstrate a need for a thorough examination of cardiac muscle to identify specific kinase(s). Obviously such activities may be important in regulating troponin activity.

The phosphorylation of a specific protein at a reasonable rate would be an important criteria for indicating a possible role for a phosphorylation reaction in a particular cellular process. We have found that TN-I is specifically phosphorylated by cyclic AMP-dependent protein kinase in the heterogeneous protein complex of troponin which is in agreement with others (3). In addition we found tropomyosin added to cardiac troponin was not phosphorylated and did not alter the maximal amount of $^{32}$P incorporated into TN-I.

The maximal rate of phosphorylation of cardiac troponin by cyclic AMP-dependent protein kinase was about 3.5 times greater than the rate of phosphorylation of unfractinated...
histones, a commonly used substrate in vitro. The rate of phosphorylation of troponin was not altered by the presence of tropomyosin. Also, the presence of actin did not appear to change significantly the rate or maximal amount of "P incorporated into cardiac troponin. More importantly, the presence or absence of Ca\textsuperscript{2+} did not significantly alter the phosphorylation of cardiac troponin in the presence of tropomyosin and actin. These results indicate that the site phosphorylated in TN-I is readily accessible to cyclic AMP-dependent protein kinase in the thin filament of the myofibrils and that the protein/protein interactions regulated by Ca\textsuperscript{2+} which turn on and turn off the thin filament do not alter the availability of the phosphorylated site.

Although Cole and Perry did not report any kinetic parameters \((K_{m}, V_{max})\) for the phosphorylation of TN-I, they found that cardiac TN-I was phosphorylated 30 times faster than white skeletal muscle TN-I under their specific conditions of incubation. In our hands, purified skeletal muscle troponin was phosphorylated at a rate of only 2% of the maximal rate of histone phosphorylation. It is significant that we found the relative rate of phosphorylation of cardiac troponin was similar to the reported rates of phosphorylation of glycogen synthase and phosphorylase \textit{a} kinase by cyclic AMP-dependent protein kinase \(24\). Since these enzymes are rapidly converted in vivo to the inactive and activated forms, respectively, upon stimulation of cyclic AMP formation, the phosphorylation of TN-I in cardiac muscle could be expected to occur rapidly in vivo. This hypothesis is supported by the observation of the rapid phosphorylation of TN-I in perfused rat hearts after epinephrine administration \(4\). This reaction may be catalyzed by cyclic AMP-dependent protein kinase since epinephrine increases cyclic AMP and activates cyclic AMP-dependent protein kinase in heart muscle \(25\).

Although cardiac troponin and skeletal muscle troponin are both composed of three distinct subunits which have functionally similar properties, the phosphorylation characteristics in vitro and in vivo demonstrate another biochemical difference between these two regulatory proteins.

Hydrogen ion concentration has not been generally recognized as an important regulatory mechanism for cyclic AMP-dependent protein kinase (see Ref. 26). However, the effect of pH on the rate of phosphorylation of cardiac troponin may be important in vivo. At an approximate intracellular pH of pH 7.0 for normal myocardium the rate of phosphorylation would be 45% of the maximal rate. A small shift in the pH in either direction would significantly alter the rate of phosphorylation of troponin which in turn may lead to the corresponding change in the inotropic state. For example, the impairment of the cardiac contractile force response to catecholamine during acidosis \(27\) may be in part due to the inhibition of the phosphorylation reaction.

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\textbf{REFERENCES}

1. Brekke, C. J., and Greiser, M. L. (1976) J. Biol. Chem. 251, 866-871
2. Reddy, Y. S., Ballard, D., Giri, N. Y., and Schwartz, A. (1973) J. Mol. Cell Cardiol. 5, 461-471
3. Cole, H. A., and Perry, S. V. (1975) Biochem. J. 149, 525-533
4. England, P. J. (1975) FEBS Lett. 50, 57-60
5. Stull, J. T. (1975) Pharmacologist 17, 234
6. Rubio, R., Bailey, C., and Villar-Palasi, C. (1975) J. Cyclic Nucleotide Res. 1, 143-150
7. Bylund, D. B., and Krebs, E. G. (1975) J. Biol. Chem. 250, 6355-6361
8. Eisenberg, E., and Kielley, W. W. (1974) J. Biol. Chem. 249, 4742-4748
9. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866-4871
10. Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971) J. Biol. Chem. 245, 1986-1995
11. Kleinsmith, L. J., Allfrey, V. G., and Mirsky, A. E. (1966) Proc. Natl. Acad. Sci. U.S.A. 55, 1182-1189
12. Hayata, K., and U. M. (1966) Clin. Chim. Acta 14, 361-366
13. Ames, B. N. (1966) Methods Enzymol. 8, 116-118
14. Weber, K., Fringle, J. R., and Ooborn, M. (1975) Methods Enzymol. 26, 3-27
15. Udenfriend, S., Stein, S., Böhlen, P., Dairmugruber, W., and Weigle, M. (1972) Science 175, 871-872
16. Solaro, R. J., Pang, D. C., and Briggs, F. N. (1971) Biochem. Biophys. Acta 245, 259-262
17. Traugh, J. A., Ashby, C. J., and Walsh, U. A. (1974) Methods Enzymol. 38C, 290-299
18. Stull, J. T., Brostrom, C. O., and Krebs, E. G. (1972) J. Biol. Chem. 247, 5272-5274
19. Krebs, E. G., Beavo, J. A., Bechtel, P. J., England, P. J., Huang, T. S., and Stull, J. T. (1973) in Exploratory Concepts in Muscle Dystrophy (Milhorat, A. T., ed) pp. 431-436, Excerpta Medica, Amsterdam
20. Perry, S. V., and Cole, H. A. (1974) Biochem. J. 141, 733-743
21. Bárány, M., Bárány, K., Gástjena, E., and Horváth, B. Z. (1973) in Exploratory Concepts in Muscle Dystrophy II (Milhorat, A. T., ed) pp. 451-462, Excerpta Medica, Amsterdam
22. Perrie, W. T., Smillie, L. B., and Perry, S. V. (1973) Biochem. J. 135, 151-164
23. Pires, E., Perry, S. V., and Thomas, M. A. W. (1974) FEBS Lett. 41, 292-296
24. Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunke- ller, P. L., Walsh, D. A., and Krebs, E. G. (1970) J. Biol. Chem. 245, 6317-6328
25. Keely, S. L., Corbin, J. D., and Park, C. R. (1975) J. Biol. Chem. 250, 4832-4840
26. Rubin, C. S., and Rosen, O. M. (1975) Ann. Rev. Biochem. 44, 831-887
27. Reynolds, R. C., and Haugnaard, N. (1967) J. Pharmacol. Exp. Ther. 156, 417-425
28. Bechtel, P. J., Beavo, J. A., Hofmann, F., Dills, W., and Krebs, E. G. (1975) Fed. Proc. 34, 671
29. Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975) J. Biol. Chem. 250, 7795-7801
30. Corbin, J. D., Keely, S. L., and Park, C. R. (1975) J. Biol. Chem. 250, 218-225
31. Hofmann, F., and Krebs, E. G. (1974) Fed. Proc. 33, 1224
32. Flescher, N., Rosen, O. M., and Neuhoff, M. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 54-58
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