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CYCLIC AMP REGULATION OF PROTEIN KINASE(S) IN RABBIT SKELETAL MUSCLE: NUCLEOSIDE TRIPHOSPHATE AND DIVALENT CATION SPECIFICITY

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SUMMARY

Two fractions of protein kinase(s) from rabbit skeletal muscle have been shown to catalyze the phosphorylation of endogenous protein, histone, and glycogen synthase I utilizing UTP, GTP, CTP, and ITP as phosphoryl donors. With one protein kinase fraction, it was found that the enzymatic activity was enhanced by Mn^{2+} when CTP served as the phosphoryl donor. This is in contrast to the inhibitory effect of Mn^{2+} on the kinase activity with ATP as the phosphoryl donor. With the second protein kinase fraction, the UTP, GTP, CTP and ITP associated phosphorylation reactions were found to be inhibited by cyclic AMP, whereas the phosphorylation reaction in which ATP served as the phosphoryl donor was found to be stimulated by cyclic AMP. These results suggest that different forms of the protein kinase(s) may exist which have different nucleoside triphosphate donor specificity and metal ion requirement. The serine residues of endogenous protein have been demonstrated to be the sites of phosphorylation when UTP, GTP, CTP and ITP served as the phosphoryl donors.

INTRODUCTION

The cyclic AMP-dependent protein kinase (EC 2.7.1.37) has been shown to exist in multiple forms with rather broad specificity, including histone, protamine, casein, fat cell lipase, ribosomal protein, troponin, glycogen synthase I and glycogen phosphorylase b kinase [1]. So far, ATP is the only nucleoside triphosphate used as phosphoryl donor. The nucleoside triphosphate specificity of the protein kinase is not entirely clear. Early in 1964, Rosell-Perez and Larner [2], using dog muscle extract, demonstrated that ATP as well as UTP could serve as substrate for the cyclic AMP-dependent glycogen synthase kinase (protein kinase). Kleinsmith and Allfrey [3] have reported that a phosphoprotein fraction isolated from calf thymus nuclei contains endogenous protein kinase activity which could utilize GTP, ITP, CTP, UTP and ATP as a phosphorylating agent with a common Michaelis constant. Greengard and

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his co-workers [4], however, provided evidence that the brain protein kinase is quite specific for ATP, as shown by the lack of inhibitory effect of other nucleoside triphosphates. However, Sanborn and Koreman [5] reported that CTP, GTP, and UTP inhibited the phosphate incorporation from ATP into histone as catalyzed by protein kinase. Labrie et al. [6] also showed that UTP, GTP, and CTP led to 45% reduction of phosphate incorporation from ATP into histone. The reason for the discrepancy among these cited results can be 2-fold. Firstly, it may be due to the difference in the properties of the protein kinase isolated from various organ sources. Secondly, it may be due to differences in the heterogeneity of the enzyme preparation.

In this preliminary communication, we report the existence of protein kinase(s) in rabbit skeletal muscle which utilize CTP, GTP, UTP, and ITP as substrates to catalyze the phosphorylation of endogenous protein and glycogen synthase I. The effects of divalent cations and cyclic AMP on the activities of these enzymes are also presented.

MATERIALS AND METHODS

Glycogen synthase I was isolated from rabbit muscle by the method of Villar-Palasi et al. [7]. [γ-^{32}P]ATP was prepared from $^{32}P_i$ following the method of Glenn and Chapel [8], and $^{32}P_i$ was obtained from New England Nuclear, Boston, Mass. [γ-^{32}P]NTP was synthesized from NDP and $^{32}P_i$ according to the method of Larner and Sanger [9], and NDP, where N referred to U, G, C, or I, was supplied by Sigma Chemical Co., St. Louis, Mo. The [γ-^{32}P]NTP was purified chromatographically with Dowex-1 column [8]. A single radioactivity spot with a corresponding R_F value for the standard NTP was observed on the Whatman No. 1 paper which had been developed chromatographically in a mixture of isobutyric acid: 1 M NH_4OH:0.1 M EDTA at pH 7.2 (10:6:0.16, by vol.). All other chemicals were of reagent grade.

Purification and assay of protein kinase(s)

Protein kinase(s) was isolated and partially purified from rabbit skeletal muscle following the procedures of Walsh et al. [10]. In the step of DEAE-cellulose column chromatography, enzymatic activities were found to be confined in two major fractions (Fraction I and II). Fraction I was eluted with 30 mM phosphate buffer, pH 7.0, containing 2 mM EDTA, while Fraction II was eluted with 300 mM phosphate buffer and 2 mM EDTA at pH 7.0. There is no detectable amount of nucleoside diphosphate kinase in either Fraction I or Fraction II under the following assay conditions.

Protein kinase(s) activity was determined isotopically according to the modified method of Reimann et al. [11], and Rebhun et al. [12]. The assay solution with a total volume of 60 μl contained glycylglycine buffer, pH 7.0, 5 μmole; $MgCl_2$ or $MnCl_2$, 0.9 μmole; histone IV or glycogen synthase I, 0.1 mg when added; and [γ-^{32}P]ATP or [γ-^{32}P]NTP, 1–10 nmoles (300 – 1200 cpm/pmole). The enzymatic reaction was initiated by adding an aliquot of Fraction I or II protein kinase(s), 30 μl, into the assay solution. After incubating at 30 °C for 10 min, 75 μl of the reaction mixture was spotted on a piece of filter paper. The spotted filter paper was first stirred in trichloroacetic acid solution, and then washed in alcohol–ether mixture and ether solvent successively. Following the final washing procedure, it was dried and then placed in a vial containing 10 ml of toluene with 0.5% PPO. The radioactivity remained on the filter paper as that of the ^{32}P-labelled protein was determined by a liquid scintillation

counter. Two kinds of blank experiments, each in duplicate, were carried out in all the experiments. In the first blank experiment, an aliquot of buffer solution was used to replace the enzyme solution in the otherwise identical reaction mixture. In the second blank experiment, the [γ-^{32}P]NTP was replaced by the acid-hydrolyzed products of [γ-^{32}P]NTP in the reaction mixture. It was found that within the limits of experimental error, both kinds of blanks gave identical results of 150–200 cpm. Protein kinase activity was expressed as pmole ^{32}P incorporated per 10 min incubation.

Hydrolysis and high-voltage electrophoresis

The enzymatic reaction described above can, after 10 min of incubation, also be terminated by the addition of 1 ml of 10% trichloroacetic acid. The ^{32}P-labelled protein precipitates were pelleted by centrifugation. The pellet was washed with trichloroacetic acid, centrifuged again, and dried. It was then hydrolyzed in 0.5 ml of 6 M HCl. Hydrolysis was carried out for 30 min at boiling temperature. The hydrolyzate, after removal of HCl, was subjected to high-voltage electrophoresis on Whatman No. 3 MM paper in a pyridine–acetate buffer, pH 4.4, for 30 min. A Savant high-voltage electrophoresis apparatus, operated at 3 kV and 180 mA, was employed. X-ray film was used to radioautography the electrophoretic patterns.

Other methods

ATP and NTP concentrations were determined enzymatically according to the method of Mills and Smith [13]. Protein concentrations of Fraction I and II kinase(s) were determined by the method of Lowry et al. [14] with crystalline bovine serum albumin as the standard.

RESULTS AND DISCUSSION

As shown in Table I (Condition 1) at a concentration of 10 mM Mg^{2+}, UTP, GTP, CTP, and ITP serve rather poorly as phosphoryl donors, when compared with ATP, for the phosphorylation of the endogenous protein catalyzed by protein kinase(s) in Fraction I. With ATP as the phosphoryl donor, cyclic AMP stimulated the reaction. In contrast, with NTP as the phosphoryl donor, cyclic AMP exerted a smaller but definite stimulatory effect. These marked differences, shown in Table I, suggest that the phosphorylation of the endogenous protein may be catalyzed by a minimum of two different forms of kinase. One form catalyzes the transfer of the terminal phosphate of the ATP molecules to endogenous protein substrate and is strongly cyclic AMP dependent, while the other catalyzes the transfer of phosphate group from NTP and is weakly cyclic AMP dependent.

Since all protein kinases require a divalent cation for activity [1], the activity of the protein kinase(s) in Fraction I was also assayed with NTP and ATP as the phosphoryl donor but with Mg^{2+} with presence of Mn^{2+} (Condition II) as well as with Mn^{2+} alone (Condition III). In Table I it shows that a partial substitution of Mg^{2+} by Mn^{2+} (Condition II) in the absence of cyclic AMP results in a general inhibition in kinase(s) activities. As Mg^{2+} is totally substituted by Mn^{2+} (Condition III), a further inhibition of the kinase(s) activity is observed. A notable exception to the general inhibition by Mn^{2+} is the observed enhancement (125%) of the kinase(s) activity with CTP as phosphoryl donor. In the presence of $1.0 \cdot 10^{-6}$ M cyclic AMP (Condition II

TABLE I

^{32}P INCORPORATION INTO ENDOGENOUS PROTEIN FROM VARIOUS NUCLEOSIDE TRIPHOSPHATES CATALYZED BY FRACTION I KINASE(S)

The incubation mixture (90 μl) consists of 5 μmole glycylglycine, pH 7.0, various concentrations of $MgCl_2$ or $MnCl_2$ as indicated, 200 μg of Fraction I protein kinase and [γ-^{32}P]NTP (ATP, 3 nmole, UTP, 5 nmole; GTP, 2 nmole; CTP; 9 nmole and ITP, 2 nmole). After 10 min incubation at 30 °C, 75 μl aliquot of the reaction mixture was spotted on paper and washed as described in Material and Methods. Ranges of values are indicated in the table.

Experimental condition	Nucleoside triphosphate added	Cation added	^{32}P incorporated (pmole/mg protein/10 min)	
			-Cyclic AMP	+Cyclic AMP ($1 \cdot 10^{-6}$ M)
I	UTP	$MgCl_2$ (10 mM)	1.35 ± 0.05	3.3 ± 0.15
	GTP		0.25 ± 0.1	0.5 ± 0.04
	CTP		0.8 ± 0.1	2.0 ± 0.1
	ITP		0.1 ± 0.05	0.3 ± 0.03
	ATP		27.9 ± 3	259.6 ± 10.0
II	UTP	$MgCl_2$ (6 mM)	0.75 ± 0.08	5.5 ± 0.5
	GTP	$MnCl_2$ (2 mM)	0 ± 0.1	0.02 ± 0.1
	CTP		1.0 ± 0.8	9.5 ± 0.8
	ITP		0.1 ± 0.01	3.1 ± 0.1
	ATP		20.7 ± 2	163.5 ± 8.0
III	UTP	$MnCl_2$ (10 mM)	0.5 ± 0.04	4.2 ± 0.03
	GTP		0 ± 0.1	0.35 ± 0.1
	CTP		1.8 ± 0.1	15.5 ± 1.0
	ITP		0.12 ± 0.05	4.1 ± 0.04
	ATP		20.0 ± 2	153.2 ± 6.0

and III), it is evident that the inhibition of the kinase(s) activity caused by the substitution of Mg^{2+} by Mn^{2+} is observed only for the phosphorylation reaction in which ATP serves as the phosphoryl donor. When the other nucleoside triphosphates served as phosphoryl donors in the presence of cyclic AMP, Mn^{2+} are generally still stimulatory. The highest stimulatory effect is observed with CTP as the phosphoryl donor.

These studies on the divalent cation specificity in the presence and absence of cyclic AMP suggest that with preference for different NTPs there are probably three forms of protein kinase(s) present in Fraction I. (1) ATP can serve as a better phosphoryl donor for a Mn^{2+}-specific and cyclic AMP-stimulated form of protein kinase. (2) GTP, ITP and UTP can serve as phosphoryl donor for a form of protein kinase which can be stimulated by Mn^{2+} in the presence of cyclic AMP, but Mg^{2+} specific in the absence of cyclic AMP. (3) CTP can serve as phosphoryl donor for a Mn^{2+}-specific and cyclic AMP-stimulated form of protein kinase.

It can be seen from Table II that as ATP served as the phosphoryl donor, the phosphorylation of the endogenous protein catalyzed by Fraction II kinase(s) is stimulated by cyclic AMP and the stimulation is considerably reduced by the substitution of Mg^{2+} by Mn^{2+}. These results are qualitatively similar to those obtained with Fraction I kinases. In the presence of histone, the Fraction II kinase(s) activity as assayed with ATP as the phosphoryl donor is increased over endogenous activity in the presence of Mn^{2+}, and the activity is further enhanced by cyclic AMP (Table II).

TABLE II

^{32}P INCORPORATION INTO VARIOUS PROTEINS FROM DIFFERENT NUCLEOSIDE TRIPHOSPHATE CATALYZED BY FRACTION II KINASE

Assay conditions were the same as described in Table I except that 64 μg of Fraction II kinase was used in each reaction mixture.

Nucleoside triphosphate	Cation	^{32}P incorporated (pmole/mg protein per 10 min)					
		No addition		Histone		Glycogen synthase	
		−Cyclic AMP	+Cyclic AMP ($1 \cdot 10^{-6}$ M)	−Cyclic AMP	+Cyclic AMP ($1 \cdot 10^{-6}$ M)	−Cyclic AMP	+Cyclic AMP ($1 \cdot 10^{-6}$ M)
ATP	$MgCl_2$ (10 mM)	21.1 ± 2	126.1 ± 10				
ATP	$MnCl_2$ (10 mM)	10.5 ± 0.08	22.4 ± 1	100 ± 8	132.1 ± 8	15.5 ± 1	30.5 ± 2
UTP	$MnCl_2$ (10 mM)	21 ± 0.2	18 ± 0.4	20 ± 0.2	10 ± 0.15	30 ± 0.15	19 ± 0.75
GTP	$MnCl_2$ (10 mM)	2.5 ± 0.08	2.0 ± 0.15	2.3 ± 0.15	1.3 ± 0.08	3.2 ± 0.6	1.9 ± 0.2
CTP	$MnCl_2$ (10 mM)	9 ± 0.05	8.3 ± 0.2	9.85 ± 0.1	7 ± 0.1	9 ± 0.1	6.8 ± 0.2
ITP	$MnCl_2$ (10 mM)	1.4 ± 0.1	0.9 ± 0.05	1.4 ± 0.1	0.6 ± 0.1	2.6 ± 0.1	1.5 ± 0.01

This increase in Fraction II kinase activity is expected, because histone not only serves as an additional phosphoryl acceptor in the assay medium but also promotes the dissociation of holoenzymes resulting in release of active forms of the enzyme [15]. Glycogen synthetase I, however, exhibits a slightly positive net effect over the endogenous protein for the ATP-phosphorylation reaction under these conditions with Mn^{2+} as divalent cation.

An interesting but totally unexpected result was obtained with the NTP-phosphorylation reaction catalyzed by Fraction II kinases. As shown in Table II,

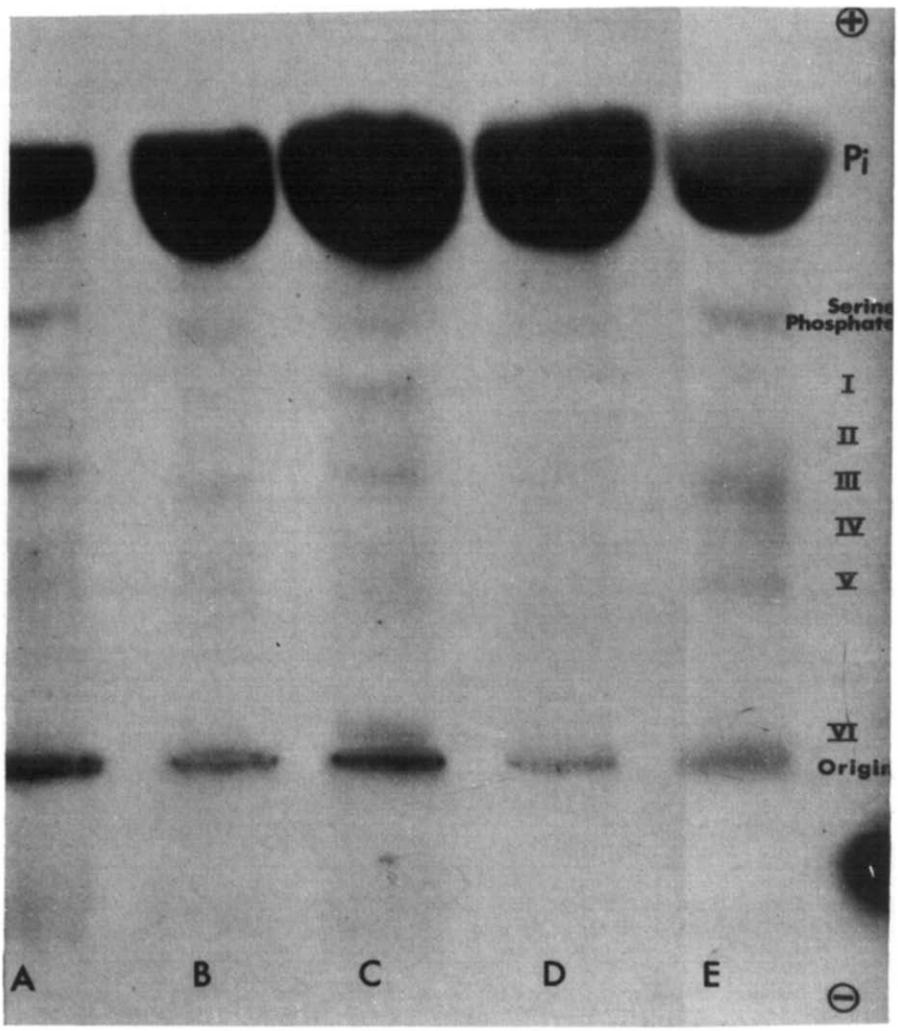

Fig. 1. Radioautogram of the partial acid hydrolyzed products of phosphorylated endogenous protein separated by electrophoresis. Fraction II (672 μg) was incubated with approximately $1.0 \cdot 10$-cpm of (A) [γ-^{32}P]UTP, (B) [γ-^{32}P]GTP, (C) [γ-^{32}P]CTP, (D) [γ-^{32}P]ITP, (E) [γ-^{32}P]ATP in the presence of 10 mM $MnCl_2$.

kinase activity is decreased by the addition of $1.0 \cdot 10^{-6}$ M cyclic AMP when NTP served as the phosphoryl donor in the presence of 10 mM Mn^{2+}. The inhibitory effect of cyclic AMP was sustained when histone and glycogen synthase were added to the assay medium, respectively. Clearly, the widely accepted mechanism that cyclic AMP promotes dissociation of holoenzymes into active catalytic subunit and a regulatory subunit–cyclic AMP complex cannot be applied to the Fraction II kinases that catalyze the NTP-phosphorylation reaction. It has been reported that the ATP-phosphorylation reaction catalyzed by a protein kinase isolated from slime mold can be inhibited by cyclic AMP [16]. Here for the first time, we observed the inhibitory effect of cyclic AMP on the NTP-phosphorylation reaction catalyzed by muscle Fraction II kinases in the presence of Mn^{2+}.

In view of the fact that ATP- and NTP-phosphorylation reactions are different in terms of the effect of cyclic AMP, comparative studies of the hydrolytic products of the phosphorylated endogenous proteins obtained with ATP and NTP as phosphoryl donor, respectively, may provide important information concerning the mechanistic differences of these reactions at the protein substrate level. The radioautograms shown in Fig. 1 demonstrate that serine phosphate is one of the major hydrolytic products of the ^{32}P-labelled protein obtained with all the ATP- and NTP-phosphorylation reactions. Other electrophoretic bands with smaller R_F values are likely to be phosphorylated dipeptide and oligopeptides resulting from the partial acid hydrolysis of the ^{32}P-labelled protein. The chemical nature of these bands were not determined. Since serine phosphate is a major hydrolytic product of all the phosphorylated protein, one can conclude that NTP-phosphorylation does, indeed, involve the formation of covalent bonding of ^{32}P-phosphate to serine moieties of the protein substrate. However, the ATP- and NTP-phosphorylation reactions cannot yet be distinguished based on the protein substrate specificity, because the electrophoretic patterns of the hydrolytic products of various phosphorylated proteins shown in Fig. 1 appeared to be rather similar.

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REFERENCES

1 Walsh, D. A. and Krebs, E. G. (1973) The Enzymes (Boyer, P. D., ed.), Vol. VIII, p. 555 Academic Press, New York
2 Rosell-Perez, M. and Larner, J. (1964) Biochemistry 3, 81
3 Kleinsmith, L. J. and Allfrey, V. G. (1969) Biochim. Biophys. Acta 175, 123
4 Greengard, P. and Kuo, J. F. (1970) Role of Cyclic AMP in Cell Function (Greengard, P. and Costa, E., eds), p. 287 Raven Press
5 Sanborn, B. M. and Koreman, S. G. (1973) J. Biol. Chem. 248, 4718
6 Labrie, F., Lemaires, S. and Courte, C. (1971) J. Biol. Chem. 246, 7293
7 Villar-Palasi, C., Rosell-Perez, M., Hizuburi, S., Huijing, F. and Larner, J. (1966) Methods in Enzymology (Neufeld, E. F. and Ginsburg, V., eds), Vol. VIII, p. 374, Academic Press, New York
8 Glenn, I. M. and Chapel, J. B. (1964) Biochem. J. 90, 147
9 Larner, J. and Sanger, F. (1965) J. Mol. Biol. 11, 491
10 Walsh, D. A., Perkins, J. P. and Krebs, E. G. (1968) J. Biol. Chem. 243, 3763

11 Reimann, E. M., Walsh, D. A. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1986
12 Rebhun, L. I. and Villar-Palasi, C. (1973) Biochim. Biophys. Acta 321, 105
13 Mills, G. T. and Smith, E. E. B. (1965) Methods of Enzymatic Analysis (Bergmeyer, H. M., ed.), p 581, Academic Press, New York
14 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. L. (1951) J. Biol. Chem. 193, 265
15 Miyamoto, E., Petzold, G. L., Kuo, J. F. and Greengard, P. (1973) J. Biol. Chem. 248, 179
16 Kuehn, G. D. (1971) J. Biol. Chem. 246, 6366