Activation of phospho*enol*pyruvate-dependent protein kinase by cytidine 5′-triphosphate in rat skeletal muscle

Roshan L. Mattoo, E. Bruce Waygood and Ramji L. Khandelwal

Department of Biochemistry, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada

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The effects of nucleotides on phospho*enol*pyruvate-dependent protein kinase in rat skeletal muscle were examined. Various ribonucleotide triphosphates were tested and the protein kinase reaction was maximally activated by cytidine 5′-triphosphate followed by uridine 5′-triphosphate and guanosine 5′-triphosphate. No activation by adenosine 5′-triphosphate was observed. Cytidine 5′-diphosphate also activated the reaction but to a significantly lesser extent. Cytidine 5′-monophosphate and cyclic cytidine 3′,5′-monophosphate showed no effect whereas cytidine was slightly inhibitory.

Protein kinase *Phospho*enol*pyruvate* *Skeletal muscle* *Phosphoprotein* *Phosphorylation* *Cytidine triphosphate*

1. INTRODUCTION

Phosphorylation of proteins and enzymes plays a significant role in the regulation of certain metabolic events in mammalian tissues [1,2]. Protein kinases, which catalyze these phosphorylation reactions, prefer ATP as the phosphate donor over other ribonucleotide triphosphates [3]. Recently, we provided evidence for a protein kinase activity in the soluble fraction of rat skeletal muscle which used phospho*enol*pyruvate (PEP) as the phosphate donor [4]. The kinase phosphorylated an endogenous protein at a serine residue. We study here the effects of various ribonucleotide triphosphates on the activity of this PEP-dependent protein kinase in rat skeletal muscle. Various nucleotides were tested and cytidine 5′-triphosphate (CTP) activated the protein kinase activity maximally in this tissue.

2. MATERIALS AND METHODS

[^{32}P]PEP was prepared as in [5]. Benzamidine, 2-(*N*-morpholino)ethanesulfonic acid (Mes), and all nucleotides were obtained from Sigma. Whatman phosphocellulose (P81) and DEAE–cellulose (DE81) papers were obtained from Fisher Scientific Co. All other reagents were of analytical grade.

2.1. *Preparation of rat tissue extracts*

Rats were killed by decapitation and skeletal muscle was immediately removed, homogenized in 4 vols of 20 mM glycylglycine (pH 7.4), 10% glycerol, 15 mM β-mercaptoethanol and 0.5 mM benzamidine. The homogenates were centrifuged at 100000 × *g* for 1 h. The clear supernatants were dialyzed overnight against the same buffer for use in these studies.

2.2. *Protein kinase activity assay*

The PEP-dependent protein kinase activity was measured as in [4]. The reaction mixture contained 25 mM Mes (pH 6.9), 0.1 mM [^{32}P]PEP (500–2000 cpm/pmol), 5 mM $MgCl_2$, 0.25 mM EGTA and crude dialyzed soluble proteins of skeletal muscle in a final volume of 0.05 ml. The addition of nucleotides was as indicated in the tables and figure legend. The reaction was started with the addition of muscle extract and was ter-

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minated after 5 min at 30°C. The radioactive protein was precipitated on a Whatman P81 paper, washed and counted for radioactivity as in [6]. One unit of enzyme activity was defined as the amount of enzyme that incorporated 1 pmol ^{32}P from [^{32}P]PEP per min.

2.3. *Analytical methods*

Protein was determined as in [7] using bovine serum albumin as the standard. PEP was separated from ribonucleotide triphosphate on DEAE–cellulose paper (Whatman DE81) as in [5].

3. RESULTS AND DISCUSSION

We have reported the presence of a PEP-dependent kinase in the soluble fraction of rat skeletal muscle [4]. During these investigations, it was shown that ATP did not participate in these phosphorylation reactions, however a more thorough investigation showed that other ribonucleotide triphosphates affected the activity of this protein kinase. The results on the effects of ATP, GTP, UTP and CTP on rat skeletal muscle PEP-dependent protein kinase are shown in table 1. At low concentration (0.1 mM), ATP showed no effect but all other nucleotides activated the reaction. The maximum activation was observed with CTP. At 1 mM, ATP and GTP inhibited the PEP-dependent protein phosphorylation whereas UTP and CTP still activated the reaction. The maximum activation was observed again with CTP.

In order to show that the activation by CTP was specific, the effects of cytidine and various cytidine nucleotides were also determined and the results are shown in table 2. The activation of PEP-dependent protein kinase was observed only with CTP and CDP; CDP was significantly less effective than CTP. CMP and cyclic 3′,5′-CMP showed no effect whereas cytidine was inhibitory. The effects of uridine nucleotides were similar to those of cytidine nucleotides but were less pronounced (not shown) and uridine showed no effect.

It was also of interest to know whether the increased phosphorylations were due to [γ-^{32}P]CTP or any other ribonucleotide triphosphates formed from [^{32}P]PEP by the following reactions CTP ⟶ CDP ⟶ [^{32}P]CTP during the incubation. For this, incubation samples were chromatographed on a DE-81 paper for the separation of ribonucleotide triphosphates from PEP [5]. There was no detectable ^{32}P-labelled ribonucleotide triphosphates formed during the reaction and in fact, the entire radioactivity was associated with PEP and a little with P_i. Further evidence against the involvement of [^{32}P]CTP in these phosphorylation reactions is provided in fig.1. If the above-mentioned reactions are operating in these incubations, then one would expect that the activation by CDP should also oc-

Table 1

Effects of ribonucleotide triphosphates on the activity of PEP-dependent protein kinase in rat skeletal muscle

Ribonucleotide triphosphate added	Concentration (mM)	Activity (units/mg protein)	Relative activity
None	–	12.8	1
ATP	0.1	12.0	0.9
	1.0	8.3	0.7
GTP	0.1	44.8	3.5
	1.0	7.4	0.6
UTP	0.1	57.6	4.5
	1.0	32.0	2.5
CTP	0.1	127.0	9.9
	1.0	141.0	11.0

Table 2

Effects of cytidine and cytidine nucleotides on the activity of PEP-dependent protein kinase in rat skeletal muscle

Compound added	Concentration (mM)	Activity (units/mg protein)	Relative activity
None	–	12.0	1
CTP	0.1	122.0	10.2
	1.0	159.0	13.2
CDP	0.1	56.4	4.7
	1.0	46.8	3.9
CMP	0.1	13.1	1.1
	1.0	11.9	1.0
Cyclic 3′,5′-CMP	0.1	11.2	0.9
	1.0	11.8	1.0
Cytidine	0.1	8.2	0.7
	1.0	8.4	0.7

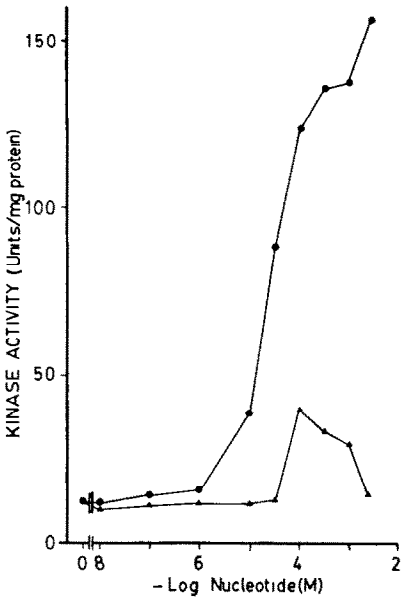

Fig.1. Effect of varying concentrations of CTP and CDP on the activity of PEP-dependent protein kinase in rat skeletal muscle. The assay conditions were as described in the text except that varying concentrations of CTP (●—●) or CDP (▲—▲) were also added in the incubation mixture.

cur at least to the same extent and probably at a lower concentration than CTP. As shown in fig.1, the activation by CDP never approached the activation observed with CTP.

Our previous results showed that PEP-dependent protein kinase phosphorylated a protein (M_r 25000) as observed on SDS–PAGE gels [4]. Later studies, using higher specific activity [^{32}P]PEP in the incubations and dried gels for autoradiographs, indicated the presence of one more phosphoprotein (M_r 43000). The presence of CTP in the incubation mixture augmented the phosphorylation of M_r 25000 protein but showed no effect on the phosphorylation of M_r 43000 protein. In addition, 3 new phosphoproteins (M_r 35000, 37000 and 59000) were also detected in the presence of CTP. These results show that PEP-dependent protein kinase activity may consist of two activities, only one of which is regulated by CTP. However, it is possible that CTP is an inhibitor of a protein phosphatase activity. Moreover, these results suggest that the PEP-dependent protein kinase may have a number of regulatory roles.

While the phosphoamino acids for each of these phosphoproteins have not been analyzed, they are probably acid-stable phosphoamino acids such as phosphoserine, phosphothreonine and phosphotyrosine as the SDS–PAGE gels were acid fixed. Experience with the phosphohistidines formed in the proteins of the PEP:sugar phosphotransferase system has shown that such would be very poorly detected under these conditions [8,9].

The metabolic connection between CTP and PEP remains obscure and at present we have no experimental explanation for these results. The only report of regulation which involves PEP and cytidine nucleotides is the activation of phospho*enol*pyruvate carboxylase by CDP in *Salmonella typhimurium* [10]. Authors in [10] explained their results as part of a balanced regulation involving aspartate inhibition of the phospho*enol*pyruvate carboxylase and the feed back inhibition of aspartate transcarbamylase by pyrimidine nucleotides. The potential involvement with pyrimidine biosynthesis in mammalian systems is thought unlikely because aspartate transcarbamylase is a large protein (M_r > 200000) and the phosphoproteins detected previously as a result of this PEP-dependent protein kinase are below M_r 100000. Secondly, the PEP-dependent protein kinase is relatively less active in liver (unpublished), in which pyrimidine biosynthesis is more commonly described. Both CTP and PEP being high energy indicators suggests that this protein kinase could be involved in the inhibition of glycolysis by the phosphorylation of glycolytic enzymes. Such possibilities are currently being investigated.

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REFERENCES

[1] Cohen, P. (1978) Curr. Top. Cell. Regul. 14, 117–196.
[2] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–959.

[3] Flockhart, D.A. and Corbin, J.D. (1982) CRC Crit. Rev. Biochem. 13, 133–186.
[4] Khandelwal, R.L., Mattoo, R.L. and Waygood, E.B. (1983) FEBS Lett. 162, 127–132.
[5] Mattoo, R.L. and Waygood, E.B. (1982) Anal. Biochem. 128, 245–249.
[6] Witt, J.J. and Roskoski, R. (1975) Anal. Biochem. 66, 253–258.
[7] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[8] Waygood, E.B. and Mattoo, R.L. (1983) Can. J. Biochem. Cell Biol. 61, 150–153.
[9] Mattoo, R.L., Khandelwal, R.L. and Waygood, E.B. (1983) Proc. Can. Fed. Biol. Soc. 26, 218.
[10] Sanwal, B.D. and Maeba, P. (1966) J. Biol. Chem. 241, 4557–4562.