STUDIES ON THE SYNTHESIS OF CDP-DIACYLGLYCEROL: STIMULATION BY GTP AND INHIBITION BY ATP AND FLUORIDE

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SUMMARY: The rat liver microsomal enzyme CTP : phosphatidate cytidylyltransferase (EC 2.7.7.41) which catalyzes the formation of CDP-diacylglycerol has been found to be markedly stimulated by GTP. The requirement for GTP is absolute, the novel GTP analogues such as guanosine 5'-[β,γ-methylene]-triphosphate, guanosine 5'-[α,β-methylene]-triphosphate, guanosine 5'-[β,γ-imido]-triphosphate and guanosine 3'-diphosphate 5'-diphosphate are without significant effect. Maximal stimulation occurs at 1 mM GTP. ATP at a concentration of 5 mM totally inhibits the formation of CDP-diacylglycerol even in the presence of optimal GTP concentration. Analogues of ATP such as adenosine 5'-[α,β-methylene]-triphosphate, adenosine 5'-[β,γ-methylene]-triphosphate and adenosine 5'-[β,γ-imido]-triphosphate are without effect on the reaction. The addition of fluoride (8 mM) likewise abolishes the stimulatory effect of GTP.

INTRODUCTION

It is now well established that the liponucleotide, CDP-diacylglycerol is a required intermediate in the synthesis of phosphatidylinositol (1), phosphatidylglycerol (2) and diphosphatidylglycerol (3) in mammalian tissues. The formation of CDP-diacylglycerol involves the reaction of CTP with phosphatidic acid and is catalyzed by CTP : phosphatidate

ABBREVIATIONS: pp[CH_2]pA, adenosine 5'-[α,β-methylene]-triphosphate; p[CH_2]ppA, adenosine 5'-[β,γ-methylene]-triphosphate; p[NH]ppA, adenosine 5'-[β,γ-imido]-triphosphate; p[NH]ppG, guanosine 5'-[β,γ-imido]-triphosphate; p[CH_2]ppG, guanosine 5'-[β,γ-methylene]-triphosphate; pp[CH_2]pG, guanosine 5'-[α,β-methylene]-triphosphate; cAMP, adenosine-3',5'-cyclic monophosphate; cGMP, guanosine-3',5'-cyclic monophosphate; ppGpp, guanosine 3'diphosphate 5'-diphosphate.

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cytidylyltransferate (EC 2.7.7.41). The properties of this enzyme as it occurs in numerous tissues and species has been reported from several laboratories (4-8).

We have been investigating the properties of CTP : phosphatidate cytidylyltransferase of rat liver and find that the formation of CDP-diacylglycerol in this tissue is stimulated several-fold by GTP and inhibited by either ATP or fluoride.

MATERIALS AND METHODS

The nucleotides ATP, GTP, dGTP, dATP, GMP, pp[CH_2]pA, p[CH_2]ppA, p[NH]ppA, p[NH]ppG, p[CH_2]ppG, pp[CH_2]pG, ppGpp, cAMP, cGMP, ITP, UTP and CTP were obtained from P.L. Biochemicals, [^{3}H]CTP from New England Nuclear, CDP-diacylglycerol (from egg phosphatidylcholine) from Serdary, the cationic detergent G3634A from Atlas Chemical Industries, Canada Ltd.

Phosphatidic acid was prepared from egg phosphatidylcholine using cabbage phospholipase D as described by Renkonen (9) and purified by silicic acid chromatography. Microsomes were prepared as described previously (10). Protein was determined by the method of Lowery et al (11) with bovine serum albumin as standard.

The assay for CDP-diacylglycerol formation was done essentially as described by Bishop and Strickland (7). Each tube contained Tris-HCl buffer pH 7.2, 100 mM; $MgCl_2$, 20 mM (added last); dithiothreitol, 4 mM; phosphatidic acid, 1 mM, emulsified in 0.5 mg of G3634A cationic detergent; [^{3}H]CTP, 1 mM (sp.act. 70,000 dpm/umole); 1.5 mg of microsomal protein in a total volume of 0.5 ml. Incubation was for 20 min. at 37°. The reaction was stopped by the addition of 4 ml of chloroform-methanol (2:1 v/v) and the amount of CDP-diacylglycerol synthesized was determined and identified as described by Bishop and Strickland (7).

RESULTS AND DISCUSSION

The effect of increasing GTP concentration on the synthesis of CDP-diacylglycerol is shown in Fig. 1. In the absence of GTP, 14 nmoles of CDP-diacylglycerol was formed, while in the presence of 1 mM GTP the activity was stimulated approximately 4-fold, resulting in the synthesis of 52 nmoles of CDP-diacylglycerol.

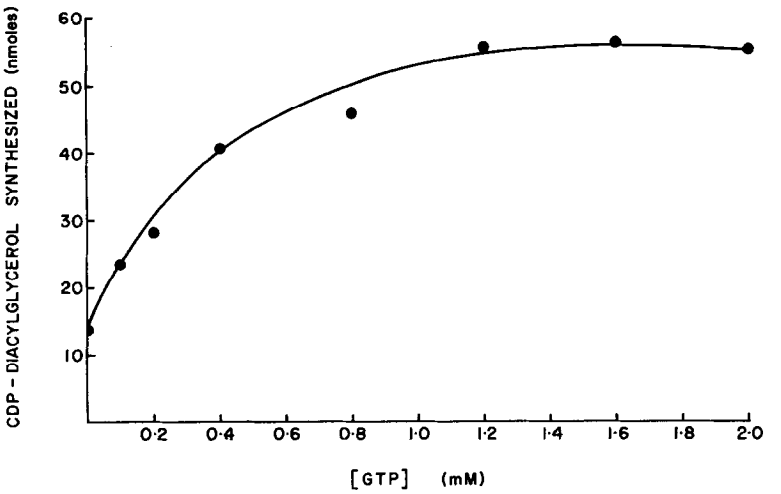

Figure 1

The activity of this rat liver microsomal enzyme in the presence of a variety of nucleotides and nucleotide analogues is shown in Table 1. GMP, ITP, cAMP and cGMP do not show any stimulatory or inhibitory effects. The deoxynucleotide dGTP was approximately 50% as effective as GTP itself, while the novel guanosine nucleotide analogues p[CH_2]ppG, pp[CH_2]pG, p[NH]ppG and ppGpp were without any significant effect.

It was found that ATP inhibited the synthesis of CDP-diacylglycerol even in the presence of optimal levels of GTP. Analogues of ATP such as pp[CH_2]pA, p[CH_2]ppA and p[NH]ppA on the other hand did not show any significant inhibitory effects (Exp. 4, Table 1). The nucleotide dATP was as effective as ATP, while AMP was without any inhibitory effect.

Besides ATP causing an inhibition of CDP-diacylglycerol synthesis, fluoride was also found to cause reversal of the

Table 1. Effect of Various Nucleotides on the Synthesis of CDP-diacylglycerol

Exp. No.	Additions	CDP-diacylglycerol Synthesized (nmoles)
1.	None	4.8
	GTP	35.0
	ITP	10.0
	UTP	7.9
	cAMP	6.3
	cGMP	5.5
	GMP	6.0
2.	None	5.8
	GTP	28.0
	ppGpp	9.5
3.	None	11.8
	GTP	44.5
	p[CH_2]ppG	13.8
	pp[CH_2]pG	10.2
	p[NH]ppG	12.9
	dGTP	25.6
4.	None	10.0
	GTP	46.7
	GTP + ATP	1.0
	GTP + p[NH]ppA	42.1
	GTP + p[CH_2]ppA	43.4
	GTP + pp[CH_2]pA	33.5
	GTP + dATP	1.0
	GTP + AMP	44.0

The concentration of all nucleotides added was 2 mM except ATP, p[CH_2]ppA, pp[CH_2]pA, p[NH]ppA, dATP and AMP which were at 5 mM. All other conditions were as described in the Materials and Methods section.

stimulatory effect resulting from GTP addition to the incubation mixture (Table 2). At a concentration of 3 mM ATP, inhibition was approximately 50%, while at 5 mM, ATP inhibition was complete.

Table 2. Effect of ATP and Fluoride on GTP-stimulated CDP-diacylglycerol Formation

Exp. No.	Additions	CDP-diacylglycerol Synthesized (nmoles)
1.	None	7.0
	GTP	36.0
	GTP + ATP (3 mM)	19.1
	GTP + ATP (5 mM)	1.0
2.	None	12.2
	GTP	57.0
	GTP + NaF (4 mM)	31.5
	GTP + NaF (8 mM)	14.5
	GTP + NaF (20 mM)	12.8
	GTP + KCl (60 mM)	56.0

The concentration of GTP was 2 mM in all experiments where shown and all other conditions were as indicated in the Methods and Materials section.

On the other hand fluoride at 4 mM inhibited to approximately 50% and at 20 mM totally reversed the GTP stimulatory effect. Increasing fluoride to 60 mM had no further inhibitory effect. As can be seen KCl (60 mM) had no effect. Increasing Mg^{2+} concentration to 40 mM, did not result in diminution of the inhibition by fluoride.

The fact that analogues of GTP did not stimulate the synthesis of CDP-diacylglycerol and that analogues of ATP did not inhibit, indicates that phosphorylation of the enzyme and/or a protein, intimately related to the enzyme, may be occurring. It is not surprising that pp[CH_2]pG and pp[CH_2]pA were not stimulatory or inhibitory respectively, since it has been shown that analogues of this type are, in some cases,

1000 times less reactive in kinase reactions than their respective triphosphates (see Ref. 12 for a review). If the synthesis of CDP-diacylglycerol by CTP : phosphatidate cytidylyltransferase is controlled by phosphorylation and dephosphorylation, the mechanism might be as follows: the enzyme could be activated by phosphorylation by a protein kinase requiring GTP. The phosphorylated active form of the enzyme could then be inactivated by a phosphoprotein phosphatase which in turn is activated by a protein kinase which specifically requires ATP for the phosphorylation of the phosphatase. The action of fluoride might be to activate a phosphoprotein phosphatase in a manner similar to that described by Najjar and Constantopoulos (13) for dephosphorylation of membrane phosphoproteins.

It has now been well documented that GTP participates in protein synthesis (14), tubulin polymerization (15), adenylate cyclase activation (16) and N-acetylglucosamine incorporation into rough microsomes (17). The above findings constitute yet another unique role for the nucleotide GTP in metabolism.

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