SYNTHESIS OF CDP-DIGLYCERIDE FROM PHOSPHATIDYLINOSITOL AND CMP

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Received July 19, 1977

Summary. A reaction in which CDP-diglyceride and inositol are formed from 1-stearoyl, 2-arachidonoyl phosphatidylinositol and CMP occurs readily in dialyzed microsomal preparations from the mouse pancreas. The reaction is Mn^{2+}-dependent, and it is inhibited by each of the two products, CDP-diglyceride and myo-inositol. It is presumed to involve the back-reaction of CDP-diglyceride:inositol phosphatidyltransferase (phosphatidyl-inositol synthetase, EC.2.7.8.11.)

CDP-diglyceride is an intermediate in the pathway of synthesis of phosphatidylinositol (1). Synthesis of CDP-diglyceride from phosphatidic acid and CTP, catalyzed by the enzyme CTP:phosphatidate cytidyltransferase (CDP-diglyceride synthetase, EC.2.7.7.41.), is the first reaction of this pathway:

$$\text{Phosphatidic acid} + \text{CTP} \rightarrow \text{CDP-diglyceride} + \text{P}\sim\text{P} \qquad \text{I}$$

The second reaction of the pathway involves the enzyme, CDP-diglyceride: inositol phosphatidyltransferase (phosphatidylinositol synthetase, EC. 2.7.8.11.), with CDP-diglyceride and inositol as substrates and phosphatidylinositol and CMP as products:

$$\text{CDP-diglyceride} + \text{Inositol} \xrightarrow{Mn^{2+}} \text{Phosphatidylinositol} + \text{CMP} \qquad \text{II}$$

In animal tissues, phosphatidylinositol has predominantly a 1-stearoyl, 2-arachidonoyl fatty acid composition (2,3). This is very different from the predominant fatty acid composition of phosphatidic acid in the tissues. It has been assumed therefore that an exchange of acyl groups in phosphatidylinositol occurs after its synthesis by the CDP-diglyceride pathway. Recently, however, CDP-diglyceride was isolated from animal tissues, and it was found also to have a predominantly 1-stearoyl, 2-arachidonoyl

ISSN 0006-291X

fatty acid composition (4,5). Its composition appears therefore to be more closely related to phosphatidylinositol than to phosphatidic acid. Thompson and MacDonald (4) suggested, as one possibility to account for their observation of this distinctive fatty acid composition, that there may be formation of CDP-diglyceride by the back-reaction of phosphatidylinositol synthetase:

$$\text{1-stearoyl, 2-arachidonoyl phosphatidylinositol + CMP} \xrightarrow{Mn^{2+}} \text{1-stearoyl, 2-arachidonoyl CDP-diglyceride + inositol} \qquad \text{III}$$

An early observation from Agranoff's laboratory suggested that CDP-diglyceride might be formed from endogenous phosphatidylinositol, but the reaction was not directly demonstrated (6). We report here evidence that reaction III occurs readily, with 1-stearoyl, 2-arachidonoyl phosphatidylinositol as an exogenous substrate, in dialyzed microsomal preparations from mouse pancreas. The work was carried out as part of an exploration of enzyme pathways which might be involved in acetylcholine-stimulated breakdown of phosphatidylinositol in the mouse pancreas (7-9).

MATERIALS AND METHODS

Materials. [5-^{3}H]cytidine 5'-monophosphate and *myo*-[2-^{3}H]inositol were from New England Nuclear, Boston, Mass. Porcine phosphatidylinositol, CDP-diolein and CDP-dicaprin were from Serdary Research Laboratories, London, Ontario, Canada. Plant phosphatidylinositol was from Supelco, Inc., Bellefonte, Pa. CDP-dipalmitin was from Sigma Chemical Co., St. Louis, Mo. ITLC-SA glass fiber sheets were from Gelman Instrument Co., Ann Arbor, Mich. CDP-diglyceride hydrolase, prepared from *Escherichia coli* membranes, was a generous gift from William Thompson and Gordon MacDonald, University of Toronto.

Preparation of dialyzed microsomes from mouse pancreas. Approximately 1g of mouse pancreas tissue was homogenized in 8 volumes of 0.25M-sucrose which contained 2.5mM-Tris maleate buffer, pH 6.0, and 0.1mM-EDTA. The homogenate was centrifuged for 10 min at 8500*g*. The supernatant fluid and the loosely pelleted microsomal material were collected and the suspension was centrifuged for 60 min at 25,000*g*. The supernatant fluid was removed and the sedimented microsomes were resuspended in 5ml of 0.01M-Tris Cl buffer, pH 8.4, and dialyzed overnight against 1 litre of the same buffer solution. All operations were carried out at 0°-4°. After dialysis, the preparations contained approximately 5mg protein per ml in a total volume of approximately 5.5ml. Incubations were carried out as described in the Tables.

Assay of [^{3}H]CDP-diglyceride. At the end of the incubation period, the reaction was stopped by the addition of 3ml of $CHCl_3$:MeOH:12M-HCl (2:1:0.01, v/v/v). The suspension was washed three times with 5ml portions of 0.1M-HCl. The aqueous phase was removed as completely as possible, and 1.3ml of MeOH were added to the combined $CHCl_3$ phase and interfacial

material. After mixing, the entire sample was taken to dryness under a stream of N_2. The residue was taken up in 50µl of $CHCl_3$:MeOH (2:1, v/v), and 25µl of a solution of commercial CDP-diglyceride (20mg per ml in $CHCl_3$:MeOH, 2:1, v/v) were added as carrier. CDP-diglyceride was separated by ascending chromatography on silicic acid-impregnated glass fiber sheets. ITLC-SA sheets were dipped in a 1% solution of potassium oxalate and air-dried. They were activated by heating at 110° for 30 min immediately before use. The samples were applied as 2cm bands and the chromatograms were developed in $CHCl_3$:MeOH:4M-NH_4OH (9:7:2, v/v/v). The CDP-diglyceride bands were located by visualization in u.v. light. They were cut out and counted in Bray's fluid in a Packard scintillation photospectrometer.

Assay of inositol. Incubations were carried out in a total volume of 0.5 ml. The reaction was stopped by the addition of 1ml of $CHCl_3$:MeOH:12M-HCl, (2:1:0.01, v/v/v), and an additional 0.5ml of H_2O were added. After centrifugation, 0.8ml of the aqueous phase were taken for inositol determination. Inositol was estimated as the trimethylsilyl derivative by gas chromatography. The method was based on that described by Majumder and Eisenberg (10).

Assay of [^{3}H]phosphatidylinositol formation from *myo*-[2-^{3}H]inositol. The dialyzed microsomal preparations were incubated as described in the Tables, and the reaction was stopped as described above. The $CHCl_3$ extracts were washed three times with 5ml portions of 0.1M-HCl. Aliquots of the $CHCl_3$ phase were counted in Bray's fluid in a Packard scintillation photospectrometer.

RESULTS

Formation of [^{3}H]CDP-diglyceride from [^{3}H]CMP and phosphatidylinositol. When dialyzed microsomal preparations from mouse pancreas were incubated in the presence of [^{3}H]CMP and Mn^{2+}, there was a small formation of [^{3}H]CDP-dilgyceride from endogenous substrate; the addition of porcine phosphatidylinositol greatly increased the amount of [^{3}H]CDP-diglyceride formed (Table 1).

In these experiments, the [^{3}H]liponucleotide product was isolated by co-chromatography with carrier CDP-diglyceride. It was further characterized as CDP-diglyceride by enzymatic hydrolysis. Samples were eluted in MeOH and subjected to hydrolysis by a partially purified preparation of CDP-diglyceride hydrolase from *Escherichia coli* (11). This enzyme hydrolyzes CDP-diglyceride to form phosphatidic acid and CMP, and the cytidine moiety then partitions into an aqueous phase. Incubations and extractions were carried out as described by Thompson and MacDonald (4). After incubation with the enzyme, 81% of the ^{3}H from experimental samples was extracted into the aqueous phase. Under the same conditions,

Table 1

Synthesis of CDP-diglyceride from [^{3}H]CMP and porcine phosphatidylinositol

Reaction mixture	[^{3}H]CDP-diglyceride formed *nmoles. mg^{-1} protein. hour^{-1}*					
	Without porcine PI			+3mM-porcine PI*		
	Mean	*SE*	*N*	*Mean*	*SE*	*N*
Basic system	1.74	±.25	20	20.9	±4.0	17
Without Mn^{2+}	0.37	±.09	2	0.52	±.09	4
Without Mn^{2+}; with 3mM-Mg^{2+}				3.24	±.12	2
With 3mM-*myo*-inositol	0.12	±.02	2	0.59	±.17	4
With 3mM-CDP-diglyceride†	1.58	±.26	3	1.49	±.69	3
With 3mM-plant phosphatidyl-inositol	1.00	±.37	2			

*1-stearoyl, 2-arachidonoyl phosphatidylinositol prepared from pig liver.
†CDP-dipalmitin, CDP-diolein, CDP-dicaprin.
Basic system: 3mM-[^{3}H]CMP, s.a. 10Ci/mole; 3mM-$MnCl_2$; 4mM-Tris Cl, pH 8.4; dialyzed pancreas microsomes, 0.8mg protein in a total volume of 0.25ml. Incubated 60 min at 37°.

commercial CDP-dipalmitin and CDP-diolein showed 80% and 82% breakdown, respectively.

The formation of [^{3}H]CDP-diglyceride in the dialyzed microsomal preparations required Mn^{2+}. When added in equimolar amounts, Mg^{2+} did not replace Mn^{2+} for this reaction (Table 1).

There was some specificity for the fatty acid composition of the phosphatidylinositol which was used as substrate for the reaction. The porcine phosphatidylinositol which was used as the substrate in the experiments shown in Table 1 was derived from pig liver, and gas chromatographic analysis indicated that it was predominantly 1-stearoyl, 2-arachidonoyl phosphatidylinositol. A sample of phosphatidylinositol which was derived from a plant source and which did not contain appreciable amounts of stearic or arachidonic acids, did not act as a substrate for [^{3}H]CDP-diglyceride formation in this system (Table 1).

The formation of [^{3}H]CDP-diglyceride in these preparations was inhibited by the addition of either *myo*-inositol or CDP-diglyceride, both of which would be the products of the back-reaction of phosphatidylinositol synthetase (reaction III). It should perhaps be mentioned, however,

Table 2

CMP-dependent formation of inositol from porcine phosphatidylinositol

Nucleotide added	Inositol formed *nmoles. mg^{-1} protein. $hour^{-1}$*		
	Mean	*SE*	*N*
CMP	20.3	±1.7	9
CDP	1.0	±0.7	2
CTP	0.4	±0.4	2
UMP	n.d		2
AMP	0.8	±0.5	2
GMP	n.d		2
CMP + 3mM-CDP-diolein	1.9	±1.1	5

Incubation system: 3mM-1-stearoyl, 2-arachidonoyl phosphatidylinositol; 3mM-$MnCl_2$; 4mM Tris Cl, pH 8.4; 3mM nucleotides, as indicated; dialyzed pancreas microsomes, 1.6mg protein in a total volume of 0.5ml. Incubated 60 min at 37°. n.d - below detection limits.

that the CDP-diglycerides which were used to inhibit were not exactly the same as the CDP-diglyceride product, since they did not have the stearoyl, arachidonoyl fatty acid composition of the phosphatidylinositol which was used as a substrate.

CMP-dependent formation of inositol from phosphatidylinositol. The other product of the back-reaction of phosphatidylinositol synthetase would be inositol. Gas chromatographic analysis of the water-soluble products of the reaction between porcine phosphatidylinositol and CMP in dialyzed microsomes showed the formation of inositol (Table 2). There was a stoichiometric relationship between the amounts of inositol and [^{3}H]CDP-diglyceride which were formed under these conditions (Tables 1 and 2). The formation of inositol was CMP-dependent. Other nucleotides which were tested - CDP, CTP, UMP, AMP and GMP - were not effective as substrates for the reaction (Table 2).

The CMP-dependent formation of inositol was inhibited by CDP-diglyceride, which would be the other product of the back-reaction of phosphatidylinositol synthetase (Table 2).

Phosphatidylinositol synthetase activity in the microsomal preparations. Since the reaction which is reported here has characteristics which would be expected for the back-reaction of phosphatidylinositol synthetase, the

Table 3

Phosphatidylinositol synthetase activity in dialyzed pancreas microsomes

Reaction mixture	[^{3}H]Phosphatidylinositol formed *nmoles. mg^{-1} protein. hour^{-1}*		
	Mean	*SE*	*N*
Complete	38.3	±2.5	3
Without CDP-diglyceride	8.5	±1.4	3
Without Mn^{2+}	1.0		1

Complete reaction mixture: 2mM-*myo*-[2-^{3}H]inositol, s.a. 10Ci/mole; 0.4mM-CDP-dipalmitin; 3mM-$MnCl_2$; 20mM Tric Cl, pH 8.4; dialyzed pancreas microsomes, 0.6mg protein in a total volume of 0.25ml. Incubated 60 min at 37°.

presence of this enzyme in the dialyzed pancreas microsomal preparations was checked. There was Mn^{2+}-dependent synthesis of [^{3}H]phosphatidylinositol from *myo*-[2-^{3}H]inositol and CDP-dipalmitin in these preparations (Table 3). This established that phosphatidylinositol synthetase activity (reaction II) was present in the preparations.

DISCUSSION

The results presented here demonstrate that the formation of CDP-diglyceride and inositol from 1-stearoyl, 2-arachidonoyl phosphatidylinositol and CMP (reaction III) can occur readily in dialyzed microsomal preparations from mouse pancreas. This reaction is the same as the back-reaction of phosphatidylinositol synthetase, which has been shown to be present in these preparations. Both reactions are Mn^{2+}-dependent, and we presume that the synthesis of CDP-diglyceride from phosphatidylinositol in this system is catalyzed by phosphatidylinositol synthetase, acting in the back direction.

The thermodynamic implications of this "cytidylolysis" of phosphatidylinositol are interesting, since the reaction results in the formation of a pyrophosphate bond in CDP-diglyceride. No nucleotide source of pyrophosphate bonds is necessary for the reaction, and it must be presumed that the energy for the formation of the phosphoric acid anhydride resides in the phosphatidylinositol molecule. This type of reaction of a

phospholipid which contains diesterified phosphate is not unknown. In their early work, Weiss, Smith and Kennedy (12) had evidence that the analogous back-reaction of phosphatidylcholine synthesis, in which CDP-choline is formed from CMP and phosphatidylcholine, could occur readily in animal tissues. Evidence for the occurrence of this reaction has since been reported from other laboratories (13,14). The well-established back-reactions of polyribonucleotide synthesis are analogous thermodynamically to these back-reactions of phospholipid synthesis. Pyrophosphate bonds are readily generated during cleavage of the phosphodiester linkages of the polymerized nucleotides.

The reaction of phosphatidylinositol and CMP to form CDP-diglyceride and inositol could account for the 1-stearoyl, 2-arachidonoyl fatty acid composition of endogenous CDP-diglyceride (4,5). Since the back-reaction cannot account for the net synthesis of CDP-diglyceride, the overall sequence would be synthesis of CDP-diglyceride from phosphatidic acid (reaction I), synthesis of phosphatidylinositol from CDP-diglyceride (reaction II), exchange of acyl groups to give 1-stearoyl, 2-arachidonoyl phosphatidylinositol, then formation of CDP-diglyceride by the back-reaction (reaction III).

The formation of CDP-diglyceride from phosphatidylinositol is also of interest in the context which led us originally to investigate it. We now have evidence that it is the pathway of acetylcholine-stimulated breakdown of phosphatidylinositol (15).

Acknowledgements. This research has received support from the National Institutes of Health, U. S. Public Health Service, Grants NS-06745, GM-00302, and 5T32-GM-07107, and from the University of Wisconsin Graduate School.

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