Identification of Cytidine Diphosphate-Diglyceride in the Pineal Gland of the Rat and Its Accumulation in the Presence of DL-Propranolol*

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SUMMARY

CDP-diglyceride, an important metabolic intermediate in the biosynthesis of phospholipids, has been isolated for the first time from a mammalian tissue. The isolated material, labeled in incubations of intact rat pineal glands with $^{32}$P, $[^3H]$cytidine, or $[^3H]$CTP in the presence of DL-propranolol, was chromatographically identical with authentic CDP-diglyceride and was able to serve as phosphatidyl donor in the enzymatic synthesis of phosphatidylinositol and phosphatidylglycerol. It yielded the expected products upon enzymatic and chemical degradation. No dCDP-diglyceride was detected.

No radioactive CDP-diglyceride was detected following incubations in the absence of propranolol. Stimulation of CDP-diglyceride labeling from $^{32}$P occurred at propranolol concentrations between 0.03 and 1.0 mM. Net synthesis of the liponucleotide was shown. At 0.1 mM, propranolol increased the incorporation of radioactivity into phosphatidyglycerol, phosphatidylinositol, and phosphatic acid. When inositol (10 mM) and propranolol (0.1 mM) were both present, phosphatidylinositol labeling was further increased, whereas stimulation of phosphatidylglycerol and CDP-diglyceride labeling was abolished.

Since CDP-diglyceride did not accumulate in the absence of the drug, its availability may normally be the limiting factor in phosphatidylinositol and phosphatidylglycerol biosynthesis. When propranolol is present, inositol may become limiting and thus may lead to the observed labeling pattern.

In previous publications we reported the stimulation of phospholipid metabolism in the pineal gland of the rat by a variety of pharmacologically active agents (1, 2). Incorporation of $^{32}$P was specifically increased into phosphatidylinositol, phosphatidylglycerol, and, to a lesser extent, phosphatic acid. The key intermediate in the biosynthetic relationship among these three lipids is the liponucleotide CDP-diglyceride as shown in the following reactions:

\[
\text{Phosphatidic acid} + \text{CTP} \rightarrow \text{CDP-diglyceride} + \text{PP}_1 \quad (1)
\]

\[
\text{CDP-diglyceride} + \text{myoinositol} \rightarrow \text{phosphatidylinositol} + \text{CMP} \quad (2)
\]

\[
\text{CDP-diglyceride} + \text{sn-glycerol-3-P} \rightarrow \text{phosphatidylglycerol-P} + \text{CMP} \quad (3)
\]

The role of GDP-diglyceride in the enzymatic synthesis of phosphatidylinositol and phosphatidylglycerol in mammalian tissues has been deduced from studies with a variety of cell-free preparations (3-8), but the intermediate itself has not been isolated from intact animal cells. Recently its occurrence in cells of Escherichia coli has been demonstrated (9).

When intact rat pineal glands were incubated with $^{32}$P in the presence of propranolol, a membrane-perturbing agent, which causes alterations in phospholipid metabolism, an unknown radioactive lipid appeared, which was not present in control incubations (X in Fig. 2C of Ref. 1). In the present study this material has been isolated and identified as CDP-diglyceride by a number of chemical and enzymatic techniques. Certain parameters influencing its accumulation have also been investigated and will be described. The findings indicate that CDP-diglyceride occurs naturally and supports the conclusion that it is the phosphatidyl donor in the biosynthesis of phosphatidylglycerol and phosphatidylinositol. Although the liponucleotide is normally present at very low levels, its accumulation can be controlled under appropriate conditions. Preliminary reports of some of these findings have been given (10).

**EXPERIMENTAL PROCEDURE**

Materials

$^{32}$P, $[^3H]$myoinositol, $[^3H]$cytidine, and $[^3H]$CTP were purchased from New England Nuclear, Boston, Mass.; CDP-glycerol

and CMP from Sigma Chemical Co., St. Louis, Mo. dl-Propranolol (Inderal) was obtained from Ayerst Laboratories, Inc., New York. Phosphatidylglycerol was either obtained commercially (bacterial, Supelco, Inc., Bellefonte, Pa.) or prepared from spinach leaves (1, 11). Those samples were used interchangeably as standards. Phosphatidylinositol was prepared from a mixture of soybean phosphatides by silicic acid chromatography.

Phosphatidylglycerol, both derived from egg lecithin, were products of Serdary Research Laboratories, London, Ontario. The preparations of CDP-diacylglyceride employed were of variable purity, but all contained authentic liponucleotide as the major constituent. One such preparation contained 5.4% phosphorus (theoretical: 6.2%) and had an A",,,,/A,, ratio = 1.89 (theoretical: 1.7). Following thin layer chromatography (1), one major ultraviolet-absorbing spot was seen. After spraying the plate with a modified Zinzadze reagent for phospholipids (12) one principal phosphorus-containing spot, coincident with the ultraviolet-absorbing area, and several minor spots were seen. Upon treatment of the commercial material with CDP-diacylglyceride hydrolase (15) for 4 hours, products were released which were identified chromatographically as phosphatidic acid and CMP.

Thin layer plates, precoated with a 250-μm layer of Silica Gel G or H (Uniplates) were obtained from Analtech, Inc., Newark, Del.

**Methods**

**Incubation of Pineal Glands**—In order to produce adequate amounts of the unknown lipid to be identified, pineal phospholipids were labeled in preparative incubations. Two to five pineal glands from 150- to 200-g female rats were incubated free floating for 3 hours at 37° in 500 μl of modified Puck’s N-16 medium (14) or in Krebs-Ringer bicarbonate buffer, pH 7.4, from which CaCl₂ and PO₄ were omitted and which contained 5 mM glucose. To both media for 1 hour in 100 μl of Krebs-Ringer bicarbonate buffer, pH 7.4, for 3 hours at 37° in 500 μl of modified Puck’s N-16 medium (14).

**Incubations with 32Pi and propranolol** was carried out, as described (13) except that Tris buffer (10 mM) was replaced by 0.1 M potassium phosphate buffer, pH 7.4. Modified Puck’s N-16 medium (14) was replaced by 0.1 mM propranolol (11), as described by Raetz et al. (15).

**Determination of Radioactivity**—Lipids and silica gel samples were counted as previously described (11). Aqueous samples were counted in Aquasol (New England Nuclear, Boston, Mass.). Radioautography of 32P-containing chromatograms was done on Kodak No-Screen NS 54 T X-ray film. For X-ray chromatograms the intensification procedure of Randerath (26) and Kodak RP Royal X-Omat (RP/R54) x-ray film were used. Paper and thin layer chromatograms were scanned with a Packard radiochromatogram scanner model 7201.

**Calculations of the extent of incorporation** were based on the specific activity of the precursor in the medium. Whether the precursor pools in the gland and the medium equilibrate rapidly is unknown. If this does not occur as to make dilution by intracellular precursor negligible, the calculations underestimate the conversion of precursor to product.

**RESULTS**

**Identification of CDP-Diglyceride**

Previous studies have established that incubations of pineals with 32P and propranolol result in substantial labeling of the unknown lipid designated X₁ (Fig. 2C in Ref. 1) which runs coincident with the ultraviolet-absorbing area, and several minor spots were seen. Upon treatment of the commercial material with CDP-diacylglyceride hydrolase (15) for 4 hours, products were released which were identified chromatographically as phosphatidic acid and CMP.

The area on the plates containing the unknown radioactive material was separated off and eluted with CHCl₃-CH₃OH-CH₂COOH-H₂O (50:20:1) and the eluate was washed with 50% aqueous methanol (15). Since the product is contaminated with small amounts of other lipids (primarily phosphatidylinositol), it was further purified by thin layer chromatography on Silica Gel H plates. The solvent used, Solvent 2, achieves good separation of CDP-diacylglyceride and phosphatidylinositol. In order to prevent degradation of the desired product, plates were washed with acetone immediately after each run and were in addition neutralized by exposure to NH₃ vapors. Elution was carried out as described by Marshall and Kates (16).

**Enzyme Reactions**—Dialyzed microsomal preparations of adult rat brain were used as source of CDP-diacylglyceride-inositol phosphatidyltransferase (17). Assays B described by these authors was used and carrier CDP-diacylglyceride was included in addition to the radioactive lipid to be tested. Bovine serum albumin was present. The reaction was stopped after 30 min with 10 volumes of CHCl₃-CH₂OH (2:1) and the solution equilibrated with 0.2 volume of 0.88% HCI (18). The upper phase was discarded, the interface was rinsed with theoretical upper phase, and carrier phosphatidylinositol was added before chromatography.

A spinach microsomal fraction was prepared and used as described by Marshall and Kates (16) for the CDP-diacylglyceride phosphatidyltransferase reaction. Carrier CDP-diacylglyceride, radioactive unknown lipid, and dl-α-glycerol-P (Sigma Chemical Co., St. Louis, Mo.) were present as substrates. The reaction was allowed to proceed for 2 hours. Phosphatidylglycerol was added to the washed lipid extract of the reaction mixture before chromatography.

CDP-diacylglyceride hydrolyase of Escherichia coli was kindly donated by Dr. C. R. H. Raetz, Harvard Medical School. The reaction was carried out as described (13) except that Tris buffer was used and the incubation was terminated after 4 hours. After partitioning, the lower phase was chromatographed with CDP-diacylglyceride and phoshatidic acid standards. CMP and dCMP were added to the upper phase before chromatography.

**Chromatography and Electrophoresis**—The following solvent systems were used for thin layer chromatography of lipids: on Silica Gel H: Solvent 1, CHCl₃-CH₂OH-CH₂COOH-H₂O (52:20:7:3); Solvent 2, CHCl₃-CH₂OH-CH₂COOH-H₂O (15:2:9:2:1); Solvent 3, CHCl₃-CH₂OH-CH₂COOH-H₂O (35:19:4:1); Solvent 4, CHCl₃-CH₂OH-CH₂COOH-H₂O (10:4:2:2:2:1); on Silica Gel G: Solvent 5, CHCl₃-CH₂OH-CH₂COOH-H₂O (25:14:2:1); Solvent 6, CHCl₃-CH₂OH-NH₂OH-H₂O (58:10:11:1); Solvent 7, diisobutylketone-CH₂COOH-H₂O (80:80:21); Solvent 8, CHCl₃-CH₂OH-NH₂OH-H₂O (50:30:7:3).

Water-soluble products of mild alkaline degradation (19, 20) were separated by high voltage electrophoresis (20, 21).

Nucleotides were chromatographed on Whatman No. 1 paper in ethanol-1 M ammonium acetate, pH 7.4 (7:3), or in 0.1 M potassium phosphate buffer, pH 6.8, saturated with ammonium sulfate-1 M propanol (30:1), as described by Raetz et al. (15).

**RESULTS**

**Identification of CDP-Diglyceride**

Previous studies have established that incubations of pineals with 32P; and propranolol result in substantial labeling of an unknown lipid designated X₁ (Fig. 2C in Ref. 1) which runs slightly slower than phosphatidylinositol in both acidic and alkaline solvent systems upon two-dimensional thin layer chromatography. In the present work, a total of six preparative incubations of pineals with 32P; and 0.1 mM propranolol was carried out in the course of the experiments to identify the unknown lipid. In the largest of these, 4.4 × 10⁶ cpm were incorporated into the total lipids (about 0.3% of the radioactivity in the medium) and the eluted and washed X₁ contained 323,000 cpm. Wasing of the eluate from silica gel with 4 N NH₄OH (15) caused losses of radioactivity into the upper phase, amounting to as much as 75%. If washings were performed with 0.5 or 1 N NH₄OH, these losses were reduced to about 20%. Paper electrophoresis of the material in the upper phase gave five radioactive bands, revealed by radioautography, three of which corresponded in RF to standards of CDP; glycerol-P, and P₁; (see also "Section c" below). The lipid in the lower phase was further purified by thin layer chromatography in Solvent 2 before its identity was examined. In control experiments, where 32P; labeled purified CDP-diglyceride was subjected to the same two step purification procedure (two-dimensional and one-dimensional chromatography, elution, and partitioning), recovery
was ~30%. Adequate radioactive product for identification was obtained by these procedures.

a. Thin Layer Chromatography—In six solvent systems the unknown cochromatographed with added CDP-diglyceride. The major band on radioautograms of the thin layer plates corresponded in size and shape to the internal standard, revealed by a sulfuric acid-chromic acid spray followed by heating at 250°. Minor bands were estimated to account for 5% or less of the radioactivity. The following RF values were observed: Solvent 1, 0.19; Solvent 2, 0.13; Solvent 3, 0.13; Solvent 5, 0.72; Solvent 6, 0.22; Solvent 7, 0.41.

b. Enzymatic Degradation—When the labeled unknown, produced in 3-hour incubations of pineal glands, was incubated with the specific CDP-diglyceride hydrolase of E. coli (13), radioactivity appeared in both the upper and lower phases of the partitioned reaction mixture. Of the 32P, 30 to 45% became glycerol. Under identical conditions alkaline methanolysis of radioactivity appeared in both the upper and lower phases of the Chloroform-rich phase was chromatographed on Solvents 4 and 8. In Solvent 8 more than 60% of the radioactivity cochromatographed with phosphatidic acid, whereas 10% remained at the origin with carrier CDP-diglyceride (Fig. 1). In the control incubation with boiled enzyme the corresponding percentages were 4 and 78. In the other solvent system the distribution of radioactivity between phosphatidic acid and CDP-diglyceride was 73% and 8% for the active and 8% and 92% for the boiled enzyme.

When the upper phase was chromatographed on paper, approximately half of the radioactive water-soluble material was present as PI (Fig. 2). The rest cochromatographed with CMP, although the presence of a very small percentage of dCMP cannot be absolutely ruled out from either the scans or radioautograms of the chromatograms. The presence of 32P in the upper phase could be due to either nonenzymatic hydrolysis of the products of enzymatic cleavage or 5'-nucleotidase or phosphatidic acid phosphatase activity in the CDP-diglyceride hydrolase preparation. The appearance of PI could be suppressed when carrier nonradioactive CDP-diglyceride was added to the labeled compound. The hydrolytic activity responsible for the formation of 32Pi (whether enzymatic or not) must therefore be very low.

c. Mild Alkaline Methanolysis—When CDP-diglyceride is converted to water-soluble compounds by mild alkaline degradation, CMP, CDP-glycerol, and glycerol-P are reported to be formed (3). Electrophoresis of the aqueous phase containing the products resulting from the hydrolysis of the 32P-labeled unknown lipid yielded a major radioactive peak corresponding to CMP and a minor one corresponding to glycerol-P. There was also a very small radioactive area corresponding to CDP-glycerol. Under identical conditions alkaline methanolysis of standard CDP-diglyceride and CDP-glycerol also yielded phosphorus-containing products primarily CMP with only a small amount of glycerol-P and CDP-glycerol. The reason why the amounts of CMP and glycerol-P were not stoichiometrically equal is not known. No appreciable amount of cyclitol-glycerol-P or PI was detected.

d. Enzymatic Synthesis of Phosphatidylinositol—In order to determine whether the unknown is capable of acting as phosphatidyl donor, it was used as labeled substrate in the CDP-diglyceride-inositol phosphatidyltransferase assay of Benjamins and Agranoff (17). The washed lipid products from the assay were chromatographed in Solvent System 2 and radioautographed (Fig. 3). Whereas in the absence of inositol essentially no radioactivity (about 1%) appeared in phosphatidylinositol, when inositol was present 42% of the added radioactivity was converted to this lipid. The rest remained with the CDP-diglyceride standard.

e. Enzymatic Synthesis of Phosphatidylglycerol—Further evidence for the ability of the unknown to donate phosphatidyl groups was obtained by incubating it with spinach microsomes which are capable of catalyzing the formation of phosphatidylglycerol (16). This synthesis requires CDP-diglyceride and glycerol-P. In the presence of glycerol-P, 79% of the added radioactivity was converted to new radioactive phospholipid. The slower running portion of the radioactive area cochromatographed with the CDP-diglyceride hydrolase from Escherichia coli and the material partitioning into the aqueous phase was chromatographed on Whatman No. 1 paper in 0.1 M potassium phosphate buffer, pH 6.8, saturated with ammonium sulfate-1-propanol (50:1). Cytidine, dCMP, CMP, and PI standards were added. The nucleotides were visualized under ultraviolet light and phosphorus-containing areas by spraying with an ammonium molybdate solution (24). Solid line, 32P; dotted line, 3H.
FIG. 3. Participation of the unknown lipid in the enzymatic synthesis of phosphatidylinositol. Incubations were carried out with rat brain microsomes according to assay B of Benjamins and Agranoff (17). The lipid substrate consisted of 0.17 mM CDP-diglyceride (CDP-DG) plus 10,000 cpm of the unknown. The lipid products of the reaction were chromatographed in Solvent System 2. The radioautogram of the chromatogram of the reaction products with and without added inositol is shown. The position of the internal standards was revealed with iodine vapors. PI, phosphatidylinositol; OR, origin.

FIG. 4. Participation of the unknown lipid in the enzymatic synthesis of phosphatidylglycerol. Incubations were carried out with spinach microsomes according to Marshall and Kates (16). The lipid substrate consisted of 0.04 mM CDP-diglyceride plus 10,000 cpm of the unknown. The lipid products of the reaction were chromatographed in two dimensions with Solvent Systems 1 and 3. The plates were washed with acetone between developments (1). Radioautograms of chromatograms of the reaction products with and without added glycerol-P are shown. PG, phosphatidylglycerol; CDP-DG, CDP-diglyceride; OR, origin.

graphed with phosphatidylglycerol (Fig. 4). The exact chemical nature of the faster running portion has not been established, but must be very closely related to that of phosphatidylglycerol. No phosphatidylglycerophosphate was detected. No reaction occurred in the absence of glycerol-P (<1%).

f. Incorporation of Cytidine and CTP—If the unknown lipid was indeed CDP-diglyceride, it appeared likely that a derivative of cytosine would be able to serve as precursor and thereby further confirm the identity of the lipid. When pineal glands were incubated under standard conditions using [3H]-labeled cytidine or CTP as radioactive precursor, virtually no radioactivity remained in the washed lipid extract. The few residual counts were probably due to a trace of precursor (about 0.005%) not removed by the wash procedure. However when 0.1 mM propranolol was present, about 0.5% of the added radioactivity was found in the lipid extract. Two-dimensional thin layer chromatography with lipid standards revealed a single radioactive spot identical in size, shape, and location to CDP-diglyceride. Approximately 5 pmol were formed from cytidine under these conditions.

Incubation of the pooled radioactive lipids from an experiment with [3H]-cytidine with the CDP-diglyceride hydrolase of E. coli resulted in the conversion of 85% of the label to watersoluble products. All of the radioactivity remaining in the chloroform phase after partition of the incubation mixture cochromatographed with CDP-diglyceride. The radioactivity scan of a paper chromatogram of the material in the aqueous phase revealed two peaks of approximately equal size (Fig. 2). One of these corresponded to CMP, the other was identified as cytidine. This was confirmed in the ethanol-1 M ammonium acetate solvent system.

Response of Pineal Phospholipid Metabolism to Changes in Propranolol Concentration

When it was discovered that drugs with β-adrenergic blocking activity were capable of selectively stimulating pineal phospholipid metabolism (1), an arbitrary concentration of 0.1 mM was used. It was of interest to examine the influence of other concentrations of the drug on the incorporation of 32P into CDP-diglyceride, phosphatidylinositol, and phosphatidylglycerol (Fig. 5). With 0.1 mM propranolol the labeling of all three lipids shown as well as of phosphatidic acid (280%) was significantly elevated as compared to control. No stimulation occurred at levels lower than 0.03 mM. The counts in phosphatidylglycerol and CDP-diglyceride in the propranolol-free controls were extremely low and variable, so that even a doubling of the incorporation was statistically not significant. In the case of CDP-diglyceride it is uncertain whether any of the labeled
compound is formed in control incubations. This is because of possible contamination due to its proximity to the highly labeled phosphatidylinositol on thin layer chromatograms. Hence where propranolol induced the labeling of CDP-diglyceride, the percentage value probably underestimates the true extent of stimulation.2

At 1 mM propranolol, only CDP-diglyceride and phosphatidic acid (790%) showed increased labeling. At this concentration, an otherwise depressive effect on phospholipid metabolism occurs with drugs which at lower concentrations stimulate phosphatidylinositol and phosphatidylglycerol labeling.3 Thus incorporation into these two lipids fell to control levels and both phosphatidylcholine and phosphatidylethanolamine labeling was reduced below 35% of control.

Figure 6. Time course of incorporation of 32P into rat pineal phospholipids in the presence of dl-propranolol. Pineal glands were incubated in quadruplicate with 32P and 0.1 mM dl-propranolol under standard conditions. The extracted lipids were separated by two-dimensional thin layer chromatography and localized by radioautography. Each point represents the mean value for four pineals. Phosphatidylcholine, ■; phosphatidic acid, △; phosphatidyglycerol, ●; phosphatidylinositol, ○; CDP-diglyceride, □.

Time Course of 32P Incorporation into Pineal Phospholipids under Influence of Propranolol

Since the rate of labeling of the individual phospholipids may be indicative of their metabolic interrelationships, the incorporation of 32P into individual phospholipids was followed for 1 hour in the presence of 0.1 mM propranolol (Fig. 6). Phosphatidic acid became labeled without a lag period, whereas the other lipids initially incorporated 32P at a slower rate. This was most pronounced for phosphatidylcholine, least for phosphatidylinositol.

1 In more recent experiments the total pineal lipid extract was partitioned with acidic wash solutions by the procedure used for preserving polyphosphoinositides (22). Under these conditions even greater incorporation of 32P into CDP-diglyceride (but into no other lipid) was observed in the presence of propranolol.

2 G. Hauser and J. Eichberg, unpublished observations.

Fig. 7. Evidence for net synthesis of CDP-diglyceride. Individual pineals were incubated for 1 hour under standard conditions with or without 0.1 mM dl-propranolol. Lipid extracts from these glands were incubated with [2-3H]inositol and rat brain microsomes according to Benjamins and Agranoff (17). The products were chromatographed in Solvent System 2 with internal standards of phosphatidylinositol and radioautograms were prepared by the intensification procedure of Randerath (23). Standards were revealed with a sulfuric acid-chromic acid spray and heat after an acetone wash to remove the scintillator and are shown to the left of the radioautogram. The products obtained with the lipid extract from a control gland (lane 1) and a gland exposed to dl-propranolol (lane 2) are shown. PI, phosphatidylinositol; I, myoinositol; OR, origin.

Net Synthesis of CDP-Diglyceride

In order to investigate whether net synthesis of CDP-diglyceride occurs under the influence of propranolol, the quantities of liponucleotide formed were assayed by means of the CDP-diglyceride-inositol phosphatidylinositol transferase reaction. The lipid substrates employed in this assay were total washed lipid extracts from pineal glands previously incubated with or without propranolol and without radioactive tracer. [2-3H]Inositol was used with the brain microsome system of Benjamins and Agranoff (17) in incubations otherwise identical with those described above for the identification of 32P-labeled CDP-diglyceride. The washed lipid extracts obtained from the incubations with microsomes were chromatographed on thin layer plates of silica gel in Solvent System 2 or by a double development in CHCl3-CH2OH-H2O (14:6:1) followed by CH3C6H4OH-NH4OH (14:6:1).

When these thin layer chromatograms were examined for radioactivity by scanning, by zonal scraping and counting of the gel samples, or by radioautography, two radioactive areas were detected. A representative pair of radioautograms is shown as Fig. 7. The lower band was identified as inositol, which had apparently been incompletely removed by the wash procedure. Labeled phosphatidylinositol was produced only in the incubations where the lipid substrate was obtained from pineal glands which had been incubated in the presence of propranolol. Prior treatment of the lipids with CDP-diglyceride hydrolase abolished
their ability to act as phosphatidy1 donor. Incubations of pineal glands with propranolol resulted in substantial accumulation of CDP-diglyceride (Table I), although the amounts of phosphatidylinositol formed in the assay undoubtedly underestimate the levels of liponucleotide. This is because our data and those of others (4, 17) show that under the conditions used less than one-half of the liponucleotide substrate is converted to phosphatidylinositol. Although labeling of phosphatidylinositol occurs also by an inositol exchange reaction when a chicken liver microsomal fraction is used as enzyme source (4), this does not occur in the assay with a brain micrososomal preparation as described by Benjamins and Agranoff (17). The amounts of either azP i or [3H]cytidine incorporated into CDP-diglyceride when pineals are incubated with the labeled precursor and propranolol (Table I) are lower than the concentration estimated with the phosphatidyltransferase assay, presumably because of the failure of the labeled and endogenous precursor pools to equilibrate.

Influence of Added Myoinositol

It seemed possible that the large increase in the incorporation of 32P i into CDP-diglyceride in the presence of propranolol might

TABLE I

CDP-diglyceride levels in rat pineal gland

The assay of CDP-diglyceride produced in pineal glands in the absence or presence of dl-propranolol was performed as described in the legend to Fig. 7. Where indicated lipids were treated with CDP-diglyceride hydrolase of Escherichia coli before being used in the assay system. Pineal gland incubations were carried out for 1 hour under standard conditions. The lipids from incubations with 32P i were separated by two-dimensional thin layer chromatography for the separation of CDP-diglyceride and the calculation was made on the basis of the observation that the distribution of label in the molecule after 1 hour is about 40% in the CMP portion and 60% in the phosphatic acid portion.

Incorporation from [G-3H]cytidine into the total washed lipid extract was taken as being all in CDP-diglyceride. Ranges are indicated and the numbers of samples are given in parentheses. Means ± S.D. for four glands are given.

<table>
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<tr>
<th>Additions</th>
<th>Phosphatidic acid</th>
<th>CDP-diglyceride</th>
<th>Phosphatidylglycerol</th>
<th>Phosphatidylinositol</th>
</tr>
</thead>
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<tr>
<td></td>
<td>pmol 32P i incorporated/pineal gland</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5.1 ± 1.0</td>
<td>0.4 ± 0.0</td>
<td>1.0 ± 0.2</td>
<td>18.9 ± 4.2</td>
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<td>dl-Propranolol, 0.1 mM</td>
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<tr>
<td>dl-Propranolol, 0.1 mM</td>
<td>5.0 ± 1.2</td>
<td>0.8 ± 0.4</td>
<td>0.9 ± 0.3</td>
<td>24.7 ± 5.0</td>
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<tr>
<td>dl-Propranolol, 0.1 mM, + myoinositol, 10 mM</td>
<td>12.5 ± 2.8</td>
<td>11.3 ± 2.2</td>
<td>12.9 ± 1.8</td>
<td>42.3 ± 3.7</td>
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<tr>
<td>dl-Propranolol, 0.1 mM, + myoinositol, 10 mM</td>
<td>9.4 ± 1.8</td>
<td>1.2 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>87.5 ± 11.2</td>
</tr>
</tbody>
</table>

a Unpublished experiments.

TABLE II

Shift by myoinositol of propranolol-induced stimulation of phospholipid metabolism

Pineal glands were incubated for 1 hour and lipids were extracted and separated as described under "Methods." Propranolol and myoinositol were present during the entire incubation period where indicated. Means ± S.D. for four glands are given.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Phosphatidic acid</th>
<th>CDP-diglyceride</th>
<th>Phosphatidylglycerol</th>
<th>Phosphatidylinositol</th>
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<td></td>
<td>pmol 32P i incorporated/pineal gland</td>
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<tr>
<td>None</td>
<td>5.1 ± 1.0</td>
<td>0.4 ± 0.0</td>
<td>1.0 ± 0.2</td>
<td>18.9 ± 4.2</td>
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<tr>
<td>Inositol, 10 mM</td>
<td>5.0 ± 1.2</td>
<td>0.8 ± 0.4</td>
<td>0.9 ± 0.3</td>
<td>24.7 ± 5.0</td>
</tr>
<tr>
<td>Propranolol, 0.1 mM</td>
<td>12.5 ± 2.8</td>
<td>11.3 ± 2.2</td>
<td>12.9 ± 1.8</td>
<td>42.3 ± 3.7</td>
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<tr>
<td>Propranolol, 0.1 mM, + myoinositol, 10 mM</td>
<td>9.4 ± 1.8</td>
<td>1.2 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>87.5 ± 11.2</td>
</tr>
</tbody>
</table>

The role of CDP-diglyceride in the biosynthesis of phospholipids has been thoroughly documented (cf. Reference 25 for review). In view of its mode of biosynthesis and further metabolism, we suggest that this compound might be more descriptively designated as phosphatidyl-CMP. The experiments which have served to elucidate the biosynthetic pathways in which it is involved were carried out in cell-free systems with exogenously added substrates, but the presence of endogenous CDP-diglyceride in mammalian tissues has not been shown. In bacteria, its existence has only recently been established in living cells of E. coli (13). The reason for the long interval between the postulate that CDP-diglyceride is an intermediate in phospholipid metabolism (3) and its isolation from intact cells is due to the extremely low steady state levels which exist under physiological conditions. The principal findings of the present study are the isolation and conclusive identification of CDP-diglyceride and the definition of conditions which bring about its accumulation in intact rat pineal glands.

The conclusion that the unknown phospholipid formed in the presence of dl-propranolol is CDP-diglyceride rests upon the following evidence: (a) identical mobility with CDP-diglyceride standards on thin layer chromatograms in six different solvent systems; (b) appearance of CMP and glycerol-P upon mild alkaline methanolysis; (c) appearance of phosphatidic acid and CMP upon hydrolysis with a bacterial enzyme preparation which is specific for the hydrolysis of CDP-diglyceride at the pyrophosphate bond; (d) ability to serve as precursor for the biosynthesis of phosphatidylinositol; (e) ability to serve as precursor for the biosynthesis of phosphatidylglycerol; (f) incorporation of cytidine and CTP into a single lipid which cochromatographs with CDP-diglyceride and yields CMP on enzymatic hydrolysis.

3H-Labeled CTP was tried as marker, because of its role as immediate precursor of CDP-diglyceride (Reaction 1, and Refs. 26 and 27). Despite its considerable negative charge it seems to be able to penetrate the pineal cell membranes suffi-
ciently well to reach the site of CDP-diglyceride biogenesis. With cytidine, which is presumably more readily taken up, several times as much product was formed. The extent of equilibration of the presursor with endogenous cytosine nucleoside and nucleotides as well as the levels of these compounds in the gland are critical in determining the magnitude of labeling; however, the quantitative aspects of neither of these factors are known. The conversion of cytidine to CTP probably proceeds by a series of three successive phosphorylations. Although the existence of a specific cytidine kinase has not yet been demonstrated in animal tissues, uridine kinase is capable of acting also on cytidine (28) and when cytidine is incubated with liver mitochondria, the so-called “salvage pathway” gives rise to mono-, di-, and triphosphates of cytidine (29). Evidence that the pineal gland can carry out these reactions rests on its ability to utilize cytidine for CDP-diglyceride synthesis (Table 1) and on the presence of a substantial fraction of \(^{32}P\) in the CDP moiety of the biosynthesized molecule (Fig. 2). An alternate pathway for the incorporation of CMP into CDP-diglyceride is the reversal of Reaction 2. If it occurs at all, this is likely to be of minor importance, especially since the preponderance of \(^{32}P\) is present on the phosphatidic acid side of the pyrophosphate bridge in the product. In contrast to the situation in E. coli (13) dCDP-diglyceride can be at most a very minor component of the pineal liponucleotide fraction.

The incorporation of \(^{32}P\) into phospholipids was stimulated at propranolol concentrations as low as 0.03 mm. At high levels (>0.3 mm) an additional effect of the drug became evident, namely reduced incorporation into phosphatidylethanolamine and phosphatidylethanolamine. The time course of labeling was very similar to that found earlier under somewhat different incubation conditions with \(\alpha\)-norepinephrine (1). The comparable features are the rapid rise of the phosphatidylinositol curve, the convex shape of the phosphatic acid curve, and the lag in labeling of phosphatidylglycerol and especially phosphatidylglycerol. The kinetics of labeling of CDP-diglyceride show a gradual accumulation of the liponucleotide despite its increased conversion to phosphatidylinositol and phosphatidylglycerol. They thus indicate that the formation of the intermediate is dramatically enhanced.

In addition to the accumulation of CDP-diglyceride, propranolol also produces a pronounced stimulation of phosphatidylglycerol labeling and a relatively much smaller increase in incorporation of \(^{32}P\) into phosphatidylinositol and phosphatic acid. Since none of these effects seems to be ascribable to changes in the ATP pool, it is likely that propranolol affects the activity of one or more of the membrane-bound enzymes which mediate phospholipid biosynthesis. The agent is known to have a pronounced influence on membranes, apparently by inducing conformational changes (30).

Insufficient information is as yet available for the formulation of a unified hypothesis which accounts for all the experimental data in terms of pool sizes and reaction rates. Although the size of the phosphatidic acid pool in pineal gland has not yet been measured, it is probably very much greater than that of CDP-diglyceride. Consequently in this tissue, as in E. coli (13), the availability of CDP-diglyceride may limit the biosynthesis of the phospholipids for which the liponucleotide serves as phosphatidyl donor. When the CDP-diglyceride pool is in creased through the action of propranolol, which at the same time enhances the labeling of phosphatidylinositol and especially of phosphatidylglycerol, another factor may become limiting. Such a factor could be that portion of free inositol which the cell can utilize for phosphatidylinositol biosynthesis. In support of this idea is the observation that the addition of exogenous inositol to propranolol-containing incubations abolishes the stimulation of phosphatidylglycerol and CDP-diglyceride labeling while causing CDP-diglyceride to be preferentially converted to phosphatidylinositol as shown in Table 11.

The accumulation of CDP-diglyceride under the influence of propranolol differs strikingly from the failure of the gland to accumulate this lipid when \(\alpha\)-norepinephrine is used as stimulating agent. The reasons for this and for the differences between the two agents in causing stimulation of phosphatidylinositol and phosphatidylglycerol labeling are not clear.

While propranolol is primarily considered a \(\beta\)-adrenergic receptor blocking agent, \(\beta\)-receptors have been shown not to be involved in the pineal phospholipid effect (1), so that this property cannot be the relevant one in explaining the observed changes. In addition to its blocking action, the compound exhibits local anesthetic (membrane-stabilizing) activity (31). It is this which appears to give rise to the observed effects, because local anesthetics are capable of altering phospholipid metabolism in a similar manner in the pineal gland (32) and do so at levels which parallel their anesthetic potency (33).

Acknowledgments—We express our gratitude to Dr. C. R. H. Raetz for his generous gift of CDP diglyceride hydrolase and to Ms. Barbara Kenney for her invaluable and skillful technical assistance.

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