The Biosynthesis of Phosphatidylinositol in Human Platelets

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ABSTRACT Homogenates of human platelets can mediate the synthesis of phosphatidylinositol from myoinositol and cytidine diphosphate diglyceride. The cytidine diphosphate diglyceride: myoinositol, phosphatidyl transferase activity is particulate-bound, and the highest specific activity is found in the membrane fraction. The production of phosphatidylinositol is decreased by sulfhydryl-binding agents, and the addition of thiols to the platelet homogenates increases the enzymatic activity. The reaction exhibits a pH optimum of 8.5–9.0. Divalent cations stimulate the reaction, and manganous chloride was the most effective of those investigated. The K_m of the enzyme for myoinositol is 0.27 mM, and the K_m for cytidine diphosphate diglyceride is 0.53 mM. The enzymatic activity of platelets isolated from patients with several diseases known to interfere with platelet clot-promoting function is similar to the enzymatic activity of platelets from normal donors.

INTRODUCTION

Human platelets rapidly incorporate radioactive phosphate, acetate, and glycerol into phospholipids (1–3). Phosphatidylinositol represents a small fraction of the phospholipids labeled with acetate and palmitate (2), but it is a significant proportion of the phospholipids labeled with glycerol (3). Moreover, in experiments using radioactive phosphate, phosphatidylinositol is intensely labeled both in vivo and in vitro (1). A selective increase in radioactive phosphate incorporation into phosphatidic acid and phosphatidylinositol occurs in leukocytes during phagocytosis as well as in a number of cells during secretion (4–8). These data suggest that phosphatidylinositol production may be important in membrane integrity and function.

Previous work in this laboratory has demonstrated that human platelets can synthesize cytidine diphosphate diglyceride (CDP diglyceride) from cytidine triphosphate and phosphatidic acid (9). When myoinositol was present during this reaction, CDP diglyceride accumulation decreased, and myoinositol-^{14}C was incorporated into a chloroform-soluble product. This suggested that CDP diglyceride was the precursor of a lipid containing myoinositol. Several laboratories (10–12) have shown that the synthesis of phosphatidylinositol can occur by the following reaction: CDP diglyceride + myoinositol → phosphatidylinositol + cytidine monophosphate (CMP). The studies reported in this paper demonstrate that human platelets contain the enzyme CDP-diglyceride: myoinositol, phosphatidyl transferase and characterize its properties.

METHODS

Separation of blood cells and preparation of homogenates. Human platelets were prepared and washed as previously described (9). Platelets, leukocytes, and erythrocytes were counted by routine techniques (13, 14).

Platelet pellets were resuspended to an estimated count of 2×10^9/ml in 0.25 M sucrose containing 10^{-3} M Na_2EDTA and 10^{-2} M 2-mercaptoethanol adjusted to pH 7.0 with NaOH (subsequently referred to as buffered 0.25 M sucrose). The platelet suspension was frozen (solid CO_2) and thawed six times, and the disrupted platelets were used as the enzyme source in most of the experiments.

Polymorphonuclear (PMN) leukocytes, lymphocytes, and erythrocytes were obtained from fresh whole blood anticoagulated with heparin, using methods which minimize platelet contamination, and they were processed as previously described (9). Except where indicated, all disrupted cells were stored at −20°C for no longer than 2 wk before they were assayed for enzymatic activity.

Preparation of CDP diglyceride. Phosphatidic acid was

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prepared from hen egg yolks, and it was reacted with cytidine-5′-monophosphomorpholidate in anhydrous pyridine to produce CDP diglyceride (15). The CDP diglyceride was purified by silicic acid chromatography and stored as the ammonium salt in aqueous solution at −20°C (9). The cytidine: phosphorus: ester molar ratio was 1.0: 2.16: 2.0, and two thin-layer chromatographic systems showed one spot (9).

Preparation of phosphatidylinositol. Isolation of phosphatidylinositol was initiated by aluminum oxide chromatography of a lipid extract of peas (16). Phosphatidylinositol was eluted with chloroform: methanol (1: 1, v/v) containing 12% H_2O. Chloroform and methanolic 0.15 N HCl were added in sufficient volume to yield two phases (17). The lower chloroform phase containing the phospholipids was passed through a short cellulose column to remove amino acids (18). Final purification was achieved by chromatography on silicic acid (19). The phosphorus: ester: nitrogen molar ratio of the phosphatidylinositol was 1.0: 2.15: 0.03.

Thin-layer chromatograms on plates of silica gel (slurred in 1 mM Na_2CO_3) developed with chloroform: methanol: acetic acid: H_2O (25: 15: 4: 2, v/v) showed one spot with an R_f of 0.52 (20). Two-dimensional thin-layer chromatograms on plates of Silica Gel G developed with chloroform: methanol: H_2O (65: 25: 4, v/v) and diisobutylketone: acetic acid: H_2O (80: 50: 10, v/v) showed a single spot with respective R_f values of 0.26 and 0.1 (21).

Following deacylation of the phosphatidylinositol with methanolic NaOH (22), 96% of the phosphate was recovered in the aqueous extract. The water-soluble product was identified as D-glycerol-1-(L-myoinositol-1-hydrogen phosphate) (GPI) by its identical migration (R_f 0.36) with an authentic sample of this substance on paper chromatograms developed with methanol: formic acid: H_2O (160: 26: 14, v/v (23) and its superimposition on the GPI peak during cochromatography on a column of Dowex 1-X10 formate (Dow Chemical Co., Midland, Mich.) (10). Recovery of phosphorus was greater than 95% by both methods, and no other phosphorus-containing substances were found.

Enzyme assay. The enzyme assay technique was similar to that employed by Carter and Kennedy (24). The reaction mixture was prepared in a 50 ml siliconized, conical-tipped glass tube, fitted with a ground-glass stopper, and maintained in melting ice. A suspension of disrupted platelets, 0.2 ml, was added to the tube followed by 0.2 ml of CDP diglyceride in Tris-HCl buffer [tris (hydroxymethyl) amino methane] (Tris) and 0.05 ml of myoinositol-2-^{3}H. The reaction mixture was brought to 37°C, and 0.05 ml of manganous chloride was added. The concentrations of the reactants used are given with each experiment. Following incubation the reaction was stopped by addition of 5 ml of methanolic 0.1 N HCl. 10 ml of chloroform was added, and the mixture was washed three times with 20 ml of 2 M KCl. The radioactivity of an aliquot of the chloroform phase was determined as previously described (9).

Chemical analyses. Protein, lipid esters, phosphorus, nitrogen, and cytidine were determined by previously described methods (9).

Materials. Myoinositol was obtained from Pfanstiehl Labs., Inc., Waukegan, Ill. D-glycerol-1-(L-myoinositol-1-hydrogen phosphate) was a product of Calbiochem, Los Angeles, Calif. Myoinositol-2-monophosphate was a product of Mann Research Labs., Inc., New York, and myoinositol-2-^{3}H (SA 3.4 Ci/mmole) was obtained from New England Nuclear Corp., Boston, Mass. Cytidine-5′-monophosphomorpholidate was a product of Sigma Chemical Co., St. Louis, Mo.

RESULTS

Preliminary experiments. When platelets suspended in buffered 0.25 M sucrose were incubated at 37°C in the presence of CDP diglyceride, myoinositol-2-^{3}H, $MnCl_2$, and Tris-HCl buffer, pH 7.5, radioactivity was found in the chloroform extract indicating the synthesis of a lipid product containing myoinositol. Platelets disrupted by repeated freezing and thawing were four times more effective in catalyzing this reaction.

Product identification. A reaction mixture identical with that described in Fig. 1, except that it contained 1 mM myoinositol-2-^{3}H (SA 7.5 Ci/mole) and 1.2 mg of platelet protein in a final volume of 1.0 ml, was incubated at 37°C for 60 min. Phosphatidylinositol 0.1 mmole was added at the end of the incubation period, and a chloroform extraction was performed as outlined in the Methods section, except that the volumes were doubled. The chloroform phase was taken to dryness under a stream of nitrogen, the residue was suspended in chloroform, and aliquots were used for thin-layer chromatography and hydrolysis.

The chloroform extract was examined for phosphatidylinositol by thin-layer chromatography as described in the Methods section. The lipids were eluted from the gel (20), and the radioactivity was determined as previously described (9). In two experiments 88% and 93% of the radioactivity migrated with the phosphatidylinositol spot. The remaining radioactivity trailed the phosphatidylinositol spot slightly in both systems, probably representing incomplete migration of the lipid.

An aliquot of the chloroform extract was taken to dryness, and the residue hydrolyzed in methanolic NaOH for 10 min at 37°C (22). After deacylation, the water-soluble products (containing 98% of the radioactivity) were examined by descending paper chromatography, using phenol: H_2O (4: 1, w/v) as the solvent, and ascending chromatography, using *n*-propanol: NH_4OH: H_2O (5: 4: 1, v/v) as the solvent (25, 26). Inositol phosphate, myoinositol, and GPI had respective Rf values of 0.05, 0.25, and 0.1 in the phenol: H_2O system, and 0.35, 0.5, and 0.6 in the *n*-propanol: NH_4OH: H_2O system. 95% of the water-soluble radioactivity after alkaline hydrolysis migrated with GPI in both systems.

The lipid formed in the reaction mixture was also hydrolyzed in 2 N HCl (19). The hydrolysis products were chromatographed on paper using the same two solvent systems. In both systems 80% of the radioactivity migrated as inositol phosphate and 15% as myoinositol; this agrees well with the data obtained by Hanahan and Olley using phosphatidylinositol isolated from beef liver (19).

Time course. The rate of formation of phosphatidylinositol was not linear over the time interval investigated

(Fig. 1). 60 min was chosen as the incubation period for the remainder of the experiments.

Enzyme concentration. The enzymatic activity varied directly with the protein concentration of the disrupted platelets up to 0.7 mg/0.5 ml of reaction mixture. At higher protein concentrations the reaction rate deviated from linearity.

pH optimum and effect of buffer salts. The enzyme was active over a wide range of pH (6.3–10.6) with an optimal activity at pH 8.5–9.0 in 0.08 M Tris-HCl (Fig. 2). Variation of the concentration of Tris-HCl from 0.04 to 0.32 M had no effect on phosphatidylinositol synthesis, but lower concentrations buffered the reaction poorly with resultant loss of enzyme activity. Boric acid-NaOH and phosphate buffers both at 0.08 M caused 60% and 34% inhibition of enzyme activity respectively (not illustrated).

Substrate and cofactors. In the absence of CDP diglyceride, divalent cation, or enzyme there was no significant production of phosphatidylinositol (Table I). The affinity of the enzyme for CDP diglyceride is shown in Fig. 3. The K_m for CDP diglyceride was 0.53 mM when calculated from a Lineweaver-Burk plot of the reciprocal of the velocity vs. the reciprocal of the substrate concentration, but it appears to be about 0.2 mM from the plot of the reaction rate against substrate concentration. The K_m of the enzyme for myoinositol was 0.27 mM when the CDP diglyceride concentration was 0.92 mM (Fig. 4). Here the K_m estimated from the plot of the reaction rate against substrate concentration agreed with that calculated from the Lineweaver-Burk plot. The velocity of the reaction using 3.6 mM myoinositol was approximately 85% of that achieved using myoinositol concentrations up to 10 mM.

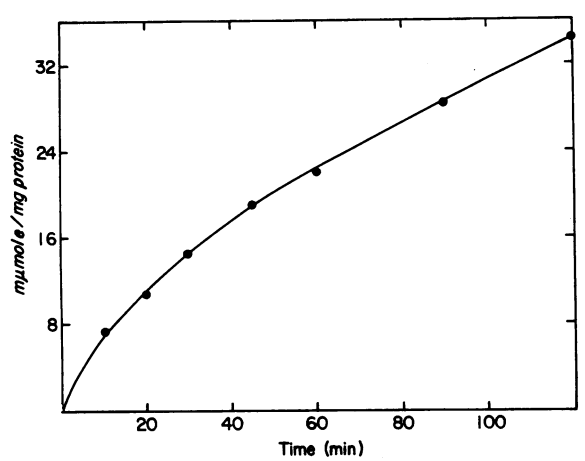

FIGURE 1 The production of phosphatidylinositol in relation to time. The reaction mixtures contained 0.6 mg of protein derived from platelets disrupted by repeated freezing and thawing, 0.1 M sucrose, 0.4 mM Na_2EDTA, 4 mM 2-mercaptoethanol, 0.92 mM CDP diglyceride, 3.6 mM myoinositol-2-^{3}H (SA 0.7 Ci/mole), 4 mM $MnCl_2$, and 0.08 M Tris-HCl buffer, pH 8.5, in a total volume of 0.5 ml. The reaction mixtures were incubated at 37°C for the time indicated.

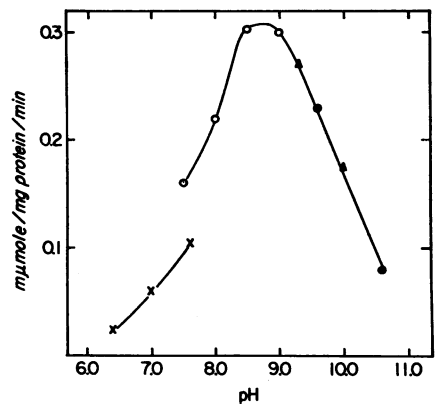

FIGURE 2 Effects of pH and buffer salts on phosphatidylinositol production. The conditions were identical with those in Fig. 1 except that the reaction mixtures contained 0.47 mg of platelet protein and were incubated for 60 min. The buffer salt and pH were varied as indicated, but the final salt concentration was always 0.08 M. X, imidazole-HCl; O, Tris-HCl; ▲, carbonate-bicarbonate; ●, glycine-NaOH.

The synthesis of phosphatidylinositol was increased by divalent cations, and $MnCl_2$ was the most effective of those investigated. Using 0.92 mM CDP diglyceride, maximal enzyme activity occurred with 3 mM $MnCl_2$. Higher concentrations of $MnCl_2$ up to 12 mM were not inhibitory. When 0.15 mM CDP diglyceride was used, maximal activity occurred with 0.8 mM $MnCl_2$, and there was no inhibition at $MnCl_2$ concentrations as high as 4 mM. Addition of $MnCl_2$ to the reaction mixture before addition of the disrupted platelets caused precipitation of the CDP diglyceride and a 90% decrease in phosphatidyl-

TABLE I

*Effect of Omission of Reagents from the Complete Reaction Mixture**

Omissions form reaction mixture	Phosphatidylinositol formed
	mμmole/mg protein/min
None	0.322
Na_2EDTA	0.338
2-Mercaptoethanol	0.140
CDP diglyceride	0.001
$MnCl_2$	0.005
Enzyme	0.0003

* The complete reaction mixtures were identical with those detailed in Fig. 1 except that they contained 0.4 mg of platelet protein and were incubated for 60 min.

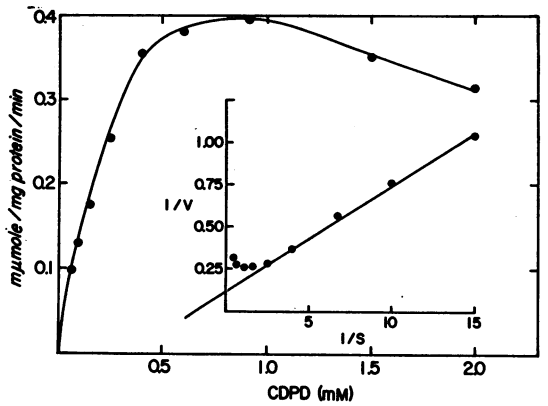

FIGURE 3 The production of phosphatidylinositol at varying concentrations of CDP diglyceride (CDPD). The conditions were identical with those detailed in Fig. 1 except that the CDP diglyceride concentration was varied as indicated, and the time of incubation was 60 min.

inositol production. $MgCl_2$ could be substituted for $MnCl_2$ but was less effective. CDP diglyceride production with 4 mM and 40 mM $MgCl_2$ was only 50% and 85% of that achieved with 4 mM $MnCl_2$. $CaCl_2$, $CuSO_4$, and $ZnSO_4$, all at 4 mM, caused no significant increase in enzymatic activity. 4 mM $CaCl_2$, in the presence of 4 mM $MnCl_2$, produced a 33% decrease in enzymatic activity.

The addition of thiols increased enzymatic activity. In the absence of Na_2EDTA, 4 mM, 10 mM, and 20 mM 2-mercaptoethanol increased phosphatidylinositol production to 250, 330, and 340%, respectively. 0.5 mM and 1.0 mM glutathione increased production to 112% and 136%, respectively.

In the absence of added 2-mercaptoethanol parachloromercuribenzoate (PCMB) 0.1 mM and 0.5 mM decreased enzyme activity by 58% and 97%. 1.25 mM and 10 mM iodoacetate caused 32% and 70% inhibition of enzyme activity in the absence of 2-mercaptoethanol. 2-mercaptoethanol reversed most of the inhibition produced by both PCMB and iodoacetate.

In the absence of 2-mercaptoethanol Na_2EDTA 0.4 mM increased enzymatic activity by 40%. This concentration of Na_2EDTA had no effect in the presence of 4.0 mM 2-mercaptoethanol. Na_2EDTA concentrations higher than 1 mM were inhibitory.

Temperature effects. The production of phosphatidylinositol was investigated at 4, 25, 37, 50, and 60°C. The enzyme activity was greatest at 37°C, and all other experiments reported were performed at this temperature. When disrupted platelets were heated to 100°C for 5 min, all enzyme activity was lost. Storage of the disrupted platelets at 4°C for 7 days resulted in a 95% loss of enzyme activity. Storage of −20°C resulted in no loss of activity after 7 days, but there was a 40% decrease in activity after 1 month.

Miscellaneous observations. There was no marked inhibition of the reaction by addition of either of the reaction products. 3.3 mM and 6.6 mM CMP produced an 11% and a 26% decrease in phosphatidylinositol production. 0.1 mM, 0.4 mM, and 2.0 mM phosphatidylinositol caused, respectively, an 8%, 12%, and 20% decrease in the reaction rate.

The enzymatic activity was not increased by several detergents. Tween-80 0.1 mg/ml and Trition X-100 2 mg/ml had no significant effect on phosphatidylinositol production. Tween-80 1 mg/ml, sodium cholate 4 mg/ml, and Cutscum 2.5 mg/ml, respectively, caused 29%, 29%, and 88% inhibition of enzyme activity. The studies with sodium cholate and Cutscum were complicated by incomplete removal of myoinositol-2-^{3}H from the chloroform extract, and the phosphatidylinositol production was corrected by appropriate controls.

Attempts to solubilize the enzymatic activity by sonication or by treating the platelet particles with organic solvents or detergents was unsuccessful. Precipitation of the platelet particles with acetone or methanol: H_2O (2:1, v/v) resulted in more than 90% loss of enzyme activity.

0.1 M NaCl, 0.04 M KCl, 0.04 M NH_4Cl, and 1 mM and 4 mM potassium fluoride had no effect on the reaction rate. Albumin 0.4 mg/ml and 2.0 mg/ml decreased phosphatidylinositol production by 2.5% and 21%.

Enzymatic activity in platelet subcellular fractions. Washed platelet pellets were homogenized as described by Marcus, Zucker-Franklin, Safier, and Ullman (27), and fractionated by differential centrifugation as previously described (9). A similar homogenate was used to prepare "membranes" and "granules," as described by

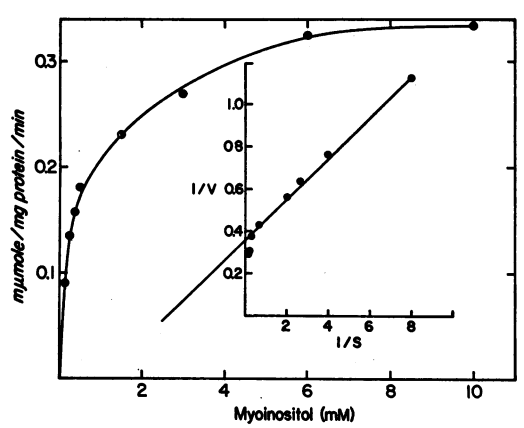

FIGURE 4 The production of phosphatidylinositol at varying concentrations of myoinositol. The conditions were identical with those detailed in Fig. 1 except that the myoinositol concentration was varied as indicated (SA 2.1 Ci/mole when myoinositol concentrations were 0.125–0.5 mM and 0.7 Ci/mole when concentrations were 1.5–10 mM). The time of incubation was 60 min.

Marcus et al. (subcellular fractions were not examined by electron microscopy). The platelet subcellular fractions were washed once and resuspended in sufficient buffered 0.25 M sucrose to make the final protein concentration 1–2 mg/ml. The 100,000 *g* pellet and the "membranes" contained the greatest specific activity (Table II). The 100,000 *g* supernate had no significant enzymatic activity and did not contain inhibitors when mixed with the other fractions.

Enzymatic activity in other blood cells. Lymphocytes and PMN leukocytes disrupted by freezing and thawing catalyzed the conversion of myoinositol-2-^{3}H into a chloroform-soluble product. The reaction rate for lymphocytes was 0.153 mμmole/mg of protein per min or 0.918 mμmole/10^8 cells per min. The values for PMN leukocytes were 0.178 mμmole/mg protein per min or 1.246 mμmole/10^8 cells per min. Erythrocyte membranes did not catalyze the conversion of myoinositol-2-^{3}H into a chloroform-soluble product. These results indicate that less than 1% of the activity present in the platelet preparations could be attributed to contamination by other cell types.

Enzymatic activity in platelets from normal subjects and patients. Platelets from 12 normal subjects disrupted by repeated freezing and thawing (protein concentration 1.2–2.4 mg/ml) catalyzed the production of 0.35–0.55 mμmole of phosphatidylinositol per mg of protein per min. The platelets of five uremic patients (blood urea nitrogen greater than 90 mg/100 ml), two patients with polycythemia vera, and one patient with Glanzmann's thrombasthenia had enzymatic activity in the same range.

TABLE II

*Enzyme Activity in Platelet Subcellular Fractions**

Platelet fraction	Phosphatidylinositol formed
	mμmole/mg protein/min
Unfractionated	0.306
1000 *g* pellet	0.221
12,000 *g* pellet	0.416
100,000 *g* pellet	1.446
100,000 *g* supernate	0.003
Membranes‡	1.320
Granules‡	0.402

* The conditions of the reactions were identical with those detailed in Fig. 1 except that 0.2–0.5 mg of protein derived from homogenized platelets was used, and the time of incubation was 60 min.

‡ These subcellular fractions were isolated on continuous sucrose gradients as described by Marcus, Zucker-Franklin, Safier, and Ullman (27).

DISCUSSION

The incorporation of myoinositol into phosphatidylinositol was first demonstrated by Agranoff, Bradley, and Brady using guinea pig kidney mitochondria (28). While investigating the increase in phosphatidylinositol synthesis caused by cytidine nucleotides, they isolated a liponucleotide which appeared to be CDP diglyceride since the water-soluble products of alkaline hydrolysis were CMP and occasionally cytidine diphosphate glycerol. They speculated that this compound might function as a precursor of phosphatidylinositol. Paulus and Kennedy synthesized CDP diglyceride and characterized an enzyme in chicken liver microsomes which catalyzed the conversion of CDP diglyceride and myoinositol to phosphatidylinositol and CMP (10). Although the enzyme was relatively specific for myoinositol, two inososes could also release CMP from CDP diglyceride at a slower rate. In the presence of saturating concentrations of manganous chloride (2 mM) some myoinositol was incorporated into phosphatidylinositol in the absence of cytidine nucleotides, and evidence that this involved an exchange reaction was presented. Similar observations have been reported during the study of phosphatidylinositol synthesis in guinea pig brain microsomes (12, 29). In guinea pig pancreas homogenates and brain microsomes, albumin was necessary for optimal enzymatic activity (11, 12).

The data presented here demonstrate that human platelets can mediate the production of phosphatidylinositol from myoinositol and CDP diglyceride. No incorporation of myoinositol-2-^{3}H into a chloroform-soluble product was observed in experiments in which CDP diglyceride was omitted. It therefore appears that no exchange reaction occurs with platelet preparations. The K_m of the enzyme for CDP diglyceride was 0.53 mM when calculated from the Lineweaver-Burk plot, but it was about 0.2 mM when estimated from the plot of the reaction rate against substrate concentration. Similar discrepancies between the K_m estimated in these two ways have been reported in other systems involving lipid emulsions (30) and remain unexplained. The K_m of 0.53 mM for CDP diglyceride is similar to those reported for guinea pig pancreas microsomes and brain microsomes when CDP-dipalmitin was used as a substrate. The fatty acid composition of the CDP diglyceride used in these experiments was not determined. Since it was synthesized from hen egg phosphatidylcholine, it would be expected to be a mixture of highly unsaturated, long-chained fatty acids (31, 32). The K_m might be different for CDP diglyceride with specific fatty acid composition. Benjamins and Agranoff have demonstrated this phenomenon in guinea pig brain microsomes (12). Similar considerations apply to the specific activity of the enzyme as discussed below. The K_m of the enzyme for

myoinositol is 0.27 mM, a value close to the K_m for myoinositol (0.2 mM) found for the enzyme present in chicken liver microsomes, but distinctly less than the K_m of the enzyme (1.5 mM) in brain and pancreas microsomes. The pH optimum of 8.5–9.0 is slightly higher than that described for the enzyme in other mammalian tissues (pH 7.5–8.6). The enzyme in platelet "membranes" has a specific activity which is about one-half the specific activity of the enzyme in pancreas microsomes, and only 6% of the specific activity of the enzyme in guinea pig brain microsomes. The high specific activity in brain microsomes is partially due to the use of the substrate CDP-didecanoin, for which the enzyme has a faster reaction rate and a lower K_m (1.7×10^{-4} M) than the other CDP diglycerides (12). The marked effect of thiol compounds and sulfhydryl-binding agents on the enzymatic activity in platelets has not been demonstrated in other mammalian tissues.

We have previously demonstrated that platelets can synthesize CDP diglyceride from phosphatidic acid and cytidine triphosphate. The enzyme characterized in this report could then convert the CDP diglyceride to phosphatidylinositol. These data provide further evidence that the rapid incorporation of radioactive phosphorus into phosphatidylinositol represents synthesis of this phospholipid at least in part and is not due solely to an exchange phenomenon. The activity of the enzyme was normal in platelets from patients with diseases known to be characterized by abnormal platelet function (uremia, polycythemia, Glanzmann's thrombasthenia). The role of this biosynthetic pathway in platelet metabolism remains to be elucidated.

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