Synthesis of ethanolamine phosphoglycerides by human platelets

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Abstract Platelet homogenates contain an ethanolaminephosphotransferase (EC 2.7.8.1) that catalyzes the synthesis of ethanolamine phosphoglycerides from cytidine-5′-diphosphate ethanolamine and 1-radyl-2-acyl-*sn*-glycerols. The enzyme is particulate-bound and requires Mn^{2+} and bile salts for optimal activity. The apparent K_m of the enzyme for cytidine-5′-diphosphate ethanolamine is 1.6×10^{-5} M when the concentration of 1,2-diacyl-*sn*-glycerols is 8.8×10^{-4} M. The pH optimum is 8.5 in Tris–HCl or glycine–NaOH buffer. The activity of the enzyme in platelets from normal subjects is 0.24–0.34 nmole/min/mg of protein.

Supplementary key words 1,2-diacyl-*sn*-glycerols · 1-alkyl-2-acyl-*sn*-glycerols · 1,2-diacyl-*sn*-glycero-3-phosphorylethanolamines · serine phosphoglycerides · 1 - alkyl-2-acyl-*sn*-glycero-3-phosphorylethanolamines · cytidine-5′-diphosphate ethanolamine:1-radyl-2-acyl-*sn*-glycerol ethanolaminephosphotransferase · erythrocyte membranes

Phosphoglycerides influence the structural and enzymatic properties of biological membranes; therefore, they are important constituents of all mammalian cells. Phosphoglycerides are of additional interest in platelets because they are involved in the clot-promoting activity of these cells. Serine and ethanolamine phosphoglycerides are particularly active in this regard (1). When human platelets are incubated with radioactive glycerol or phosphate in vitro, radioactive serine and ethanolamine phosphoglycerides are produced, suggesting that de novo synthesis of these lipids occurs in platelets (2, 3). Some of the enzymes involved in the synthesis of phosphoglycerides by human platelets have been investigated (4–6).

In mammalian tissues the synthesis of serine phosphoglycerides occurs by exchange of serine for the ethanolamine or choline constituent of ethanolamine or choline phosphoglycerides (7–10). Thus, the final step in the de novo synthesis of ethanolamine phosphoglycerides and an intermediary step in the synthesis of a portion of serine phosphoglycerides is accomplished by the enzyme cytidine-5′-diphosphate ethanolamine:1-radyl-2-acyl-*sn*-glycerol ethanolaminephosphotransferase (EC 2.7.8.1). The present studies demonstrate the presence of ethanolaminephosphotransferase in human platelets and define some of the properties of the enzyme.

METHODS

Preparation of cell homogenates

Platelets, lymphocytes, polymorphonuclear leukocytes, and erythrocyte membranes were separated from fresh whole blood as previously described (11). Cells and membranes were suspended in 0.25 M sucrose containing 1 mM disodium ethylenediaminetetraacetate (Na_2EDTA), pH 7.0, and 0.01 M 2-mercaptoethanol. Unless indicated, the suspensions were sonicated, and the protein concentration of each was adjusted to 2–3 mg/ml (12). Separation of platelets from individual patients was modified by using as anticoagulant 1 vol of 0.13 M trisodium citrate for 9 vol of whole blood.

Chromatography and quantitative analysis of lipids

Silicic acid column chromatography was performed on SilicAR cc-4, 100–200 mesh (Mallinckrodt Chemical Works, St. Louis, Mo.). Silica gels with and without $CaSO_4$ binder (Camag, Inc., Milwaukee, Wis.) were used for thin-layer chromatography. Lipid glycerol was determined by hydrolysis of neutral lipids with 2 N HCl and conversion of the glycerol to formaldehyde with $NaIO_4$ (13). Glyceryl ethers were determined by hydrolysis of neutral lipids with 2 N KOH in 95% ethanol, extraction into diethyl ether, and production of formaldehyde with $NaIO_4$ (14). Lipid esters were determined as previously described (11).

Reagents

Choline phosphoglycerides were prepared from the lipids of egg yolks by column chromatography on aluminum

Abbreviations: diacylglycerols, 1,2-diacyl-*sn*-glycerols; alkylacylglycerols, 1-alkyl-2-acyl-*sn*-glycerols; diacyl-GPE, 1,2-diacyl-*sn*-glycero-3-phosphorylethanolamines; alkylacyl-GPE, 1-alkyl-2-acyl-*sn*-glycero-3-phosphorylethanolamines; alkenylacyl-GPE, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphorylethanolamines.

oxide and silicic acid (11). Ethanolamine phosphoglycerides were separated from egg lipids by chromatography on diethylaminoethyl cellulose (DE 23, Reeve Angel, Clifton, N.J.) (15). Disodium adenosine-5′-triphosphate (equine muscle, Na_2ATP), cytidine-5′-monophosphoric acid (CMP), sodium cytidine-5′-triphosphate (type 1, Na CTP), sodium cytidine-5′-diphosphate ethanolamine (Na CDP ethanolamine), sodium 3-phospho-D-glycerate (Na phosphoglycerate), ethanolamine dihydrogen phosphate, L-serine, sodium nicotinamide adenosine dinucleotide phosphate (NADP), and polyoxyethylene sorbitan monolaurates (Tween 20 and Tween 80) were obtained from Sigma Chemical Co., St. Louis, Mo. *sn*-Glycero-3-phosphorylethanolamine was obtained from Supelco, Inc., Bellefonte, Pa. Sodium glycodeoxycholate was obtained from Calbiochem, La Jolla, Calif. Octylphenoxypolyethoxyethanol (Triton X-100) was obtained from Packard Instrument Co., Downers Grove, Ill.

Preparation of radioactive CDP ethanolamine

CDP [1,2-^{14}C]ethanolamine was prepared by incubating [1,2-^{14}C]ethanolamine dihydrogen phosphate (New England Nuclear, Boston, Mass.) with a preparation of CTP:ethanolaminephosphate cytidylyltransferase (EC 2.7.7.14) that had been concentrated by ammonium sulfate fractionation of the supernatant fraction of a rat liver homogenate (16). The reaction mixture contained 5 ml of the enzyme preparation suspended in 0.02 M Tris–HCl, pH 7.5, 2 ml of 0.1 M Tris–succinate, pH 6.0, 2.2 mM Na CTP, 2.2 mM Na_2ATP, 12 mM Na phosphoglycerate, 0.22 mM ethanolamine dihydrogen phosphate, 1.14 mM [1,2-^{14}C]ethanolamine dihydrogen phosphate (sp act 22 Ci/mole), and 12 mM $MgCl_2$ in a final volume of 10 ml. The mixture was incubated at 37°C for 120 min and then placed in a boiling-water bath for 5 min. The reaction mixture was filtered, and the volume of the filtrate was raised to 30 ml with water. The CDP [1,2-^{14}C]ethanolamine was purified by chromatography on Dowex-1-formate 2X, 200–400 mesh, using a linear gradient of 0 to 0.06 N formic acid. Samples containing water were counted in 10 ml of Aquasol (New England Nuclear) in a Tri-Carb 3003 liquid scintillation spectrometer (Packard). The radioactive fractions containing CDP ethanolamine were pooled, concentrated by lyophilization, and rechromatographed in the same manner. The amount of CDP ethanolamine was determined by ultraviolet spectrophotometry at 280 nm (17). The yield of CDP [1,2-^{14}C]ethanolamine (based on the recovered radioactivity) was 94%, and the specific activity was 19.1 Ci/mole. The product was chromatographed on paper using as solvent systems 95% ethanol–1 M ammonium acetate saturated with sodium tetraborate 7:3 (v/v) and *n*-butanol–acetic acid–water 5:2:3 (v/v/v). In both systems, 99.8% of the radioactivity migrated in the same position as CDP ethanolamine and 0.2% migrated in the same position as ethanolamine dihydrogen phosphate (16, 17).

Lipid substrates

Diacylglycerols were prepared by incubation of choline phosphoglycerides with phospholipase C (*Clostridium welchii*, grade B, Calbiochem) (12). Alkylacylglycerols were prepared by incubation of the lipids of dogfish (*Squalus acanthias*) liver (Kent Laboratories, Ltd., Vancouver, B.C.) with hog pancreas lipase (steapsin, Nutritional Biochemicals Corp., Cleveland, Ohio) (18). Diacylglycerols and alkylacylglycerols were isolated by column chromatography on silicic acid using mixtures of hexane and diethyl ether (12). Diacylglycerols were eluted with hexane–diethyl ether 7:3 (v/v) and had an ester:glycerol ratio of 1.95:1. Thin-layer chromatography on silica gel (containing $CaSO_4$) using as solvent systems benzene–diethyl ether–ethanol–acetic acid 50:40:2:0.2 (by vol) and hexane–diethyl ether–acetic acid 80:20:1 (v/v/v) showed single spots with R_f values of 0.58 and 0.17, respectively (19, 20). No 1,3-diacyl-*sn*-glycerols were detected in freshly prepared samples. Alkylacylglycerols were recovered with hexane–diethyl ether 85:15 (v/v) and 7:3 (v/v); final purification was achieved by preparative thin-layer chromatography on silica gel (without binder) using as solvent hexane–diethyl ether 7:3 (v/v). The ester:glyceryl ether ratio was 1.1:1. Thin-layer chromatography of the alkylacylglycerols (performed as for the diacylglycerols) gave single spots with R_f values of 0.62 and 0.20, respectively. Diolein and monoolein were obtained from Sigma Chemical Co.

Other procedures

Phosphoglycerides were subjected to mild alkaline and acid hydrolysis, and the water-soluble products were identified by paper chromatography (21). Lipid emulsions were prepared by sonication of mixtures of lipid, detergent, and buffer salts immediately prior to each experiment (12). Protein was determined as previously described (11).

Enzyme assay

The standard reaction mixture contained 0.3 mM CDP [1,2-^{14}C]ethanolamine (sp act 2.8 Ci/mole), 80 mM Tris–HCl, pH 8.0, 0.88 mM diacylglycerols, 0.82 mM sodium glycodeoxycholate, 10 mM $MnCl_2$, 0.1 M sucrose, 0.4 mM Na_2EDTA, 4 mM 2-mercaptoethanol, and enzyme in a final volume of 0.5 ml. The mixtures were incubated under air at 37°C in 50-ml glass-stoppered centrifuge tubes. Unless indicated, the reactions were stopped by the addition of 5 ml of methanolic 0.1 N HCl. Chloroform (10 ml, containing 0.1 μmole of ethanolamine phosphoglycerides) was added, and the chloroform extract was washed three times by shaking with 20 ml of 2 M KCl (22). An aliquot of the chloroform phase was dried, and the radioactivity was determined with a liquid scintillation spectrome-

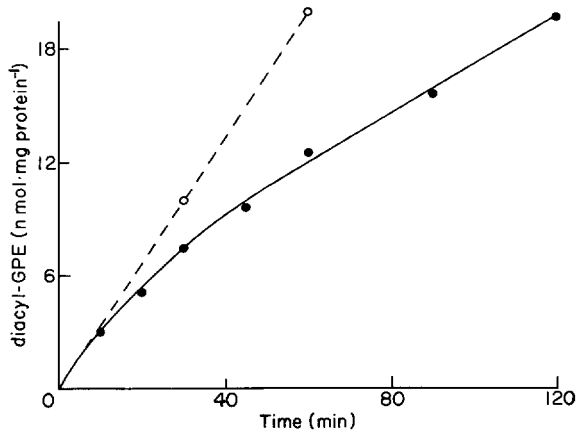

Fig. 1. Production of diacyl-GPE at different time intervals. Reaction mixtures contained 0.4 mg of platelet protein and were incubated for the times indicated. ●, data from complete reaction mixtures as described in the Methods section; ○, data from complete reaction mixtures made 0.8 mM in ethanolamine phosphoglycerides.

ter using 2,4-bis-[2-(5-*tert*-butylbenzoxazolyl)]-thiophene as scintillator (11). The quantity of ethanolamine phosphoglycerides that was synthesized was calculated by dividing the radioactivity detected by the specific activity of the CDP [1,2-^{14}C]ethanolamine.

RESULTS

Identification of product

Reaction mixtures containing 0.46 mg of platelet protein were prepared as described in the Methods section. After incubation at 37°C for 30 min, 5 ml of methanol (without HCl) was added to each reaction mixture, and a chloroform extraction was performed as described. The washed chloroform extracts were reduced in volume under a stream of nitrogen; aliquots were used for thin-layer chromatography as well as for mild alkaline and acid hydrolysis. Thin-layer chromatography was carried out on plates coated with silica gel (slurried in 1 mM Na_2CO_3) developed with chloroform–methanol–acetic acid–water 25:15:4:2 (by vol) and on plates coated with silica gel (containing $CaSO_4$ binder) developed with chloroform–methanol–7 N NH_4OH 65:35:5 (v/v/v). The results showed that 96–98% of the radioactivity in the chloroform extracts migrated as ethanolamine phosphoglycerides (R_f 0.75 and 0.47, respectively [23, 24]). No other areas of localized radioactivity were present. After alkaline hydrolysis and extraction, 97% of the radioactivity was present in the aqueous phase. After mild acid hydrolysis of the remaining lipids, 1% of the radioactivity was present in the aqueous extracts and 2% was present in the chloroform phase. The water-soluble, deacylated products of both alkaline and acid hydrolysis migrated to the same position as *sn*-glycero-3-phosphorylethanolamine, as determined by ascending paper chromatography using as solvents *n*-propanol–NH_4OH–water 5:4:1 (v/v/v) and methanol–formic acid–water 80:13:7 (v/v/v) (R_f 0.57 and 0.50, respectively) (25). The chloroform-soluble, deacylated product migrated in the same position as lysophosphatidylethanolamine during subsequent thin-layer chromatography (performed as described above for diacyl-GPE) and was probably 1-alkyl-*sn*-glycero-3-phosphorylethanolamine. The results of similar experiments in which no lipid substrate was added are recorded in **Table 1.** The addition of alkylacylglycerols decreased the synthesis of diacyl-GPE and increased the synthesis of alkylacyl-GPE and of alkenylacyl-GPE. The amount of alkenylacyl-GPE produced was confirmed by thin-layer chromatography of the lipid extract. The lipid was applied to the silica gel plates, which were exposed to HCl fumes to hydrolyze the alkenyl groups, and subsequently the plates were developed with solvents (26). The product of acid hydrolysis migrated in the same position as lysophosphatidylethanolamine and was probably 2-acyl-*sn*-glycero-3-phosphorylethanolamine.

TABLE 1. Effect of addition of 1-radyl-2-acyl-*sn*-glycerols on production of ethanolamine phosphoglycerides

Addition to Reaction Mixture	Diacyl-GPE	Alkenylacyl-GPE	Alkylacyl-GPE
	nmoles/min/mg protein		
Buffer	0.0399	0.0034	0.0057
Diacylglycerols and glycodeoxycholate	0.3100	0.0037	0.0073
Alkylacylglycerols and glycodeoxycholate	0.0109	0.0117	0.2190

Reaction mixtures contained 0.46 mg of platelet protein and were incubated for 30 min. Tris–HCl, 0.08 M, pH 8, or the same buffer containing 0.82 mM sodium glycodeoxycholate and 0.88 mM 1-radyl-2-acyl-*sn*-glycerols was added as indicated; otherwise, reaction mixtures were identical with those described in the Methods section. Reaction mixtures were incubated at 37°C for 30 min. Reactions were stopped by adding 5 ml of methanol.

Time course

The synthesis of diacyl-GPE was not linear with respect to time after 10 min (**Fig. 1**). In reaction mixtures containing 0.4 mg of platelet protein, 1.7% of the diacylglycerols and 5.2% of the CDP ethanolamine were incorporated into diacyl-GPE during an incubation of 120 min. The addition of choline or ethanolamine phosphoglycerides to the reaction mixtures (so that the final concentration of each phosphoglyceride was 0.8 mM) before incubation (but not after incubation) increased the amount of radioactive diacyl-GPE recovered. When the incubation mixtures were made 0.8 mM in ethanolamine phosphoglycerides, the reaction rates were linear for 60 min (Fig. 1). When radioactive ethanolamine phosphoglycerides (0.4 mM, sp act 12 mCi/mole) were added to incubation mixtures containing 0.4 mg

TABLE 2. Effect of omission of components from the complete reaction mixture

Omission from Reaction Mixture	Ethanolamine Phosphoglyceride Produced
	nmoles/min/mg protein
None	0.320
2-Mercaptoethanol	0.315
Glycodeoxycholate	0.049
Diacylglycerols	0.045
Diacylglycerols and glycodeoxycholate	0.043
Enzyme	0.000
Mn^{2+}	0.000

Time of incubation was 30 min, and 0.46 mg of platelet protein was present except where indicated; otherwise, complete reaction mixtures were identical with those described in the Methods section.

of platelet protein and no CDP ethanolamine, 8% of the radioactivity (equivalent to 16 nmoles of diacyl-GPE) was not chloroform-soluble after incubation for 30 min. There was no decrease in the radioactivity recovered from similar reaction mixtures containing platelet protein that had been heated in a boiling-water bath for 5 min. It is concluded that the addition of phosphoglycerides to the reaction mixtures prevented the breakdown of radioactive diacyl-GPE by competing for a phospholipase present in the platelet homogenates. Subsequent reaction mixtures were incubated for 30 min without added phosphoglycerides.

Detergent effects

Detergents were utilized because the synthesis of ethanolamine phosphoglycerides was not increased by the addition of diacylglycerols alone (**Table 2**). In incubation mixtures containing 0.8 mg of platelet protein, the production of ethanolamine phosphoglycerides was 0.038 nmole/min/mg of protein without detergents. Incubation mixtures made 0.4 mg/ml in sodium glycodeoxycholate (0.82 mM) gave reaction rates of 0.285 nmole/min/mg of protein. Concentrations of sodium glycodeoxycholate above 1.23 mM were less effective, and 4.10 mM sodium glycodeoxycholate inhibited the enzyme (reaction rate of 0.010 nmole/min/mg of protein). Sodium taurodeoxycholate, sodium deoxycholate, sodium glycocholate, and Triton X-100 (all 0.4 mg/ml) were, respectively, 112, 103, 88, and 21% as effective as 0.4 mg/ml sodium glycodeoxycholate. Tween 20 and Tween 80 (both 0.4 mg/ml) did not alter the reaction rate obtained without detergents (0.038 nmole/min/mg of protein). Sodium dodecyl sulfate and cetyltrimethylammonium bromide (both 0.4 mg/ml) inhibited the enzyme (reaction rates of 0.002 and 0.000 nmole/min/mg of protein, respectively).

Enzyme concentration

No radioactivity could be extracted with chloroform from reaction mixtures that did not contain platelet homogenates, and no ethanolamine phosphoglycerides were produced by platelet homogenates that had been heated for 5 min in a boiling-water bath. The synthesis of diacyl-GPE did not increase in direct proportion to the amount of platelet homogenate added to the reaction mixture (**Fig. 2**). The sigmoidal curve suggests inhibition of the enzyme at lower protein concentrations. Two further observations confirm that this is caused by sodium glycodeoxycholate. When heat-inactivated platelets were added to different amounts of platelet homogenate so that the final amount of protein was 0.5 mg per assay, the synthesis of diacyl-GPE was directly proportional to the amount of active platelet homogenate (not illustrated). Addition of bovine albumin instead of heat-inactivated platelets did not affect the enzyme inhibition noted using amounts of platelet protein of 0.1–0.3 mg per assay. Secondly, when incubation mixtures were prepared so that a constant ratio of 1 μmole of sodium glycodeoxycholate per mg of protein was maintained, the synthesis of diacyl-GPE was linear with respect to the amount of protein up to 0.625 mg per assay (Fig. 2).

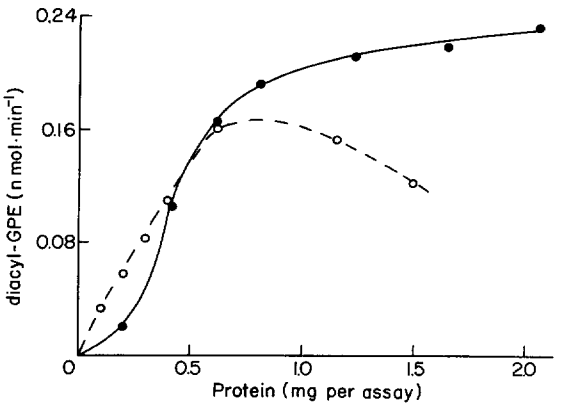

Fig. 2. Production of diacyl-GPE at different protein concentrations. Reaction mixtures contained platelet protein in the amount indicated and were incubated for 30 min. ●, data from complete reaction mixtures as described in the Methods section; ○, data from complete reaction mixtures with a constant ratio of 1 μmole of sodium glycodeoxycholate/mg of protein.

Disruption of platelets by sonication resulted in a 40% increase in enzyme activity, but repeated freezing and thawing (solid CO_2–acetone bath) caused a 20% decrease in enzyme activity. Platelets were routinely disrupted by sonication prior to each experiment (12).

pH optimum and effect of buffer salts

The enzyme was active over the pH range of 7–9, and the reaction rate was greatest at pH 8.5 (**Fig. 3**). Glycine–NaOH, Tris–HCl, and triethanolamine–HCl (all 0.08 M) were effective buffers at pH 8.5. Incubation of reaction mixtures buffered with 0.04 M Tris–HCl, pH 8.5, for 30 min resulted in a decrease in the pH of the mixtures to 7.7 and in a 31% decrease in the reaction rate. Incubation mixtures buffered with 0.20–0.40 M Tris–HCl, pH 8.5, gave reaction rates that were 21–32% less than the rates

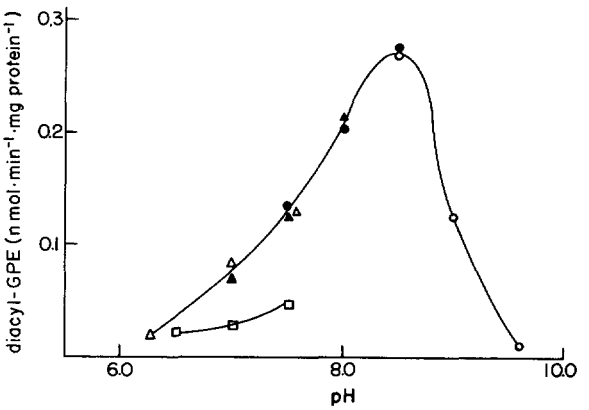

Fig. 3. Effect of pH and buffer salts on production of diacyl-GPE. Conditions were identical with those described in the Methods section. Reaction mixtures contained 0.6 mg of platelet protein and were incubated for 30 min. Buffer salt concentration was maintained at 0.08 M but the pH was varied as indicated. ○, glycine–NaOH; ●, Tris–HCl; ▲, triethanolamine–HCl; △, imidazole–HCl; □, KH_2–K_2HPO_4.

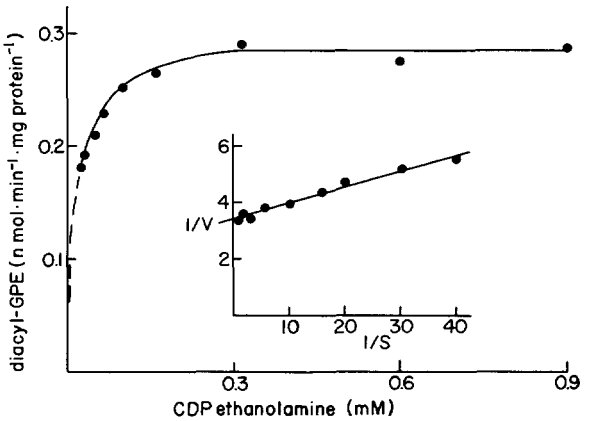

Fig. 5. Production of diacyl-GPE at different concentrations of CDP ethanolamine. Conditions were identical with those described in the Methods section except the concentration of CDP [1,2-^{14}C]ethanolamine (sp act 5.6 Ci/mole) was varied as indicated. Reaction mixtures contained 0.5 mg of platelet protein and were incubated for 30 min.

obtained in similar mixtures containing 0.08 M Tris. Potassium phosphate buffer (0.08 M) inhibited the enzyme at pH 7 and 7.5 (Fig. 3). The monovalent cations Na^+ (100 mM) and K^+ (40 mM) did not alter the reaction rate.

Substrates and cofactors

Small amounts of ethanolamine phosphoglycerides were produced in the absence of mixtures of diacylglycerols and sodium glycodeoxycholate (Tables 1 and 2). The reaction rates determined from these preparations incubated for 30 min were 20% less than the rates determined from 2-min incubations. In reaction mixtures containing sodium glycodeoxycholate and diacylglycerols the synthesis of diacyl-GPE was increased eightfold (Tables 1 and 2). **Fig. 4** shows the reaction rates obtained by changing the amount of diacylglycerols while maintaining a constant concentration of platelet protein (0.5 mg/assay) and sodium glycodeoxycholate (0.82 mM). Using the data from incubation mixtures containing concentrations of diacylglycerols of 0.15 mM or more, the reaction rate was one-half of the maximal velocity at 0.3 mM diacylglycerols, as determined graphically from the plot of the reciprocal of the reaction rate vs. the reciprocal of the substrate concentration (Fig. 4). Substitution of 0.88 mM diolein for the diacylglycerols increased the reaction rate to 0.38 nmoles/min/mg of protein. Monoolein, 0.9 mM (in the presence or absence of sodium glycodeoxycholate), decreased the reaction rate to 0.01 nmole/min/mg of protein, indicating inhibition of the enzyme. Substitution of 0.88 mM alkylacylglycerols for diacylglycerols produced a 38-fold increase in the synthesis of alkylacyl-GPE (Table 1).

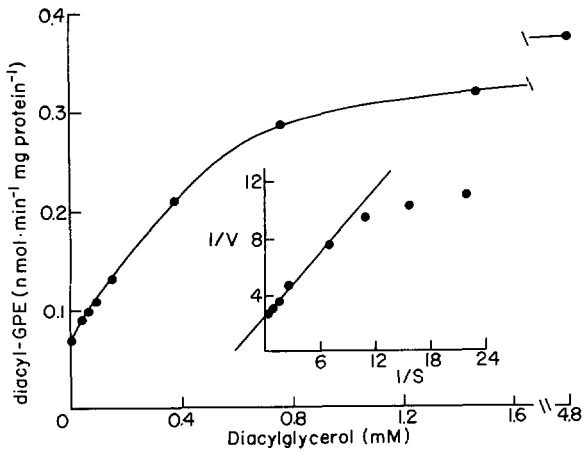

Fig. 4. Production of diacyl-GPE at different concentrations of diacylglycerols. Conditions were identical with those described in the Methods section except the concentration of diacylglycerols was varied as indicated. Reaction mixtures contained 0.5 mg of platelet protein and were incubated for 30 min.

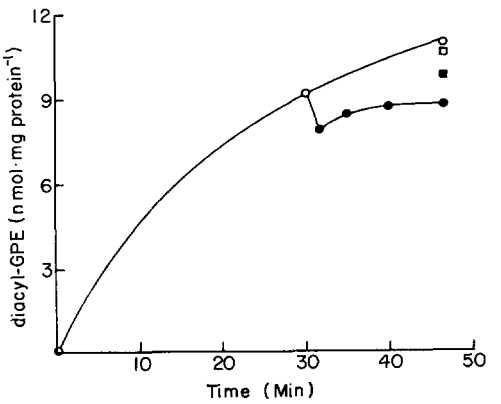

Fig. 6. Effect of L-serine on synthesis of diacyl-GPE. Reaction mixtures were identical with those detailed in the Methods section and contained 0.4 mg of platelet protein. After incubation for 30 min, 0.05 ml of water, 0.05 ml of 20 mM $CaCl_2$, or 0.05 ml of 0.5 M L-serine plus 20 mM $CaCl_2$ (pH 8) was added. After mixing, incubations were continued for the time indicated. ○, no addition; ●, $CaCl_2$ and L-serine; □, water; ■, $CaCl_2$.

As illustrated in the plot of the reciprocal of the reaction rate vs. the reciprocal of the concentration of substrate, the apparent K_m of the enzyme for CDP ethanolamine was 1.6×10^{-5} M when the concentration of diacylglycerols was 0.88 mM (**Fig. 5**).

Divalent cation was necessary for enzyme activity, and Mn^{2+} was the most effective cation examined. The optimal concentration of $MnCl_2$ was 10 mM, but the reaction rate was decreased by only 6% at concentrations of 5 and 16 mM. $FeSO_4$ and $CoCl_2$ (both 10 mM) were, respectively, 30 and 7% as effective as 10 mM $MnCl_2$. $MgCl_2$, 50 and 10 mM, gave reaction rates that were, respectively, 18 and 8% of that obtained with $MnCl_2$. No enzyme activity could be demonstrated in the presence of $CaCl_2$, $ZnSO_4$, and $CuSO_4$ (all 10 mM). In the presence of $MnCl_2$, the reaction rate was decreased by 61% by making the incubation mixture 10 mM in $CaCl_2$.

Temperature effects

Reaction mixtures containing 0.5 mg of platelet protein were incubated for 30 min at various temperatures, and the reaction rates were compared. Reaction mixtures incubated at 4°C, 22°C, 30°C, 45°C, and 65°C resulted in reaction rates of 9, 47, 72, 82, and 8%, respectively, of the reaction rate of mixtures incubated at 37°C. Platelet homogenates stored at −20°C for 7 and 54 days lost 4 and 39% of their initial activity, respectively. Incubation of platelet homogenates at 37°C for 30 min caused a 6% decrease in enzyme activity, but homogenates incubated with mixtures made 1.1 mM in diacylglycerols and 1.0 mM in sodium glycodeoxycholate at 37°C for 30 min lost 85% of their initial enzyme activity. Cell homogenates were routinely used immediately after preparation or were stored at −20°C for no longer than 7 days.

Miscellaneous observations

No inhibition of enzyme activity could be demonstrated in the presence of the product, diacyl-GPE. The addition of CMP, 0.3 and 0.9 mM, decreased the reaction rate by 27 and 62%, respectively. Separation and washing of platelets at pH 6.5 using Tris–HCl, Tris–maleate, or imidazole–HCl buffer inhibited the enzyme by 21–32% in comparison with platelets processed in Tris–HCl buffer at pH 7.5. Addition of thiols, including glutathione (1.28 and 6.4 mM), 2-mercaptoethanol (4, 10, and 30 mM), and dithiothreitol (1.28 and 6.4 mM) did not affect the reaction rate. Na_2EDTA and 2-mercaptoethanol were used in the preparation of platelet homogenates because they affect the stability of other platelet enzymes.

Effect of L-serine and Ca^{2+}

The addition of L-serine and Ca^{2+} to reaction mixtures after an incubation period of 30 min caused a rapid decrease in diacyl-GPE that was probably due to the conversion of ethanolamine phosphoglycerides to serine phosphoglycerides by base exchange (**Fig. 6**).

TABLE 3. Conversion of alkylacyl-GPE to alkenylacyl-GPE

Omission from Reaction Mixture	Diacyl-GPE	Alkenylacyl-GPE	Alkylacyl-GPE
		dpm	
None	84	206	1860
NADP	76	314	1720
Platelet homogenate	49	78	2080

The substrate (1-radyl-2-acyl-GPE) was isolated from reaction mixtures in which alkylacylglycerols were used for the synthesis of ethanolamine phosphoglycerides (Table 1). The lipid was suspended in buffer by sonication. Complete mixtures contained 0.35 mg of platelet protein, 0.1 M sucrose, 10 mM Na_2ATP, 0.4 mM Na_2EDTA, 4 mM 2-mercaptoethanol, 0.16 mM 1-radyl-2-acyl-GPE (sp act 15 mCi/mole), 0.08 M Tris–HCl, pH 7.4, 5 mM $MgCl_2$, and 2 mM NADP in a final volume of 0.5 ml. Mixtures were incubated at 37°C for 30 min, and reactions were stopped by the addition of 5 ml of methanol. Chloroform extractions were performed as indicated in the Methods section, and products were identified by sequential mild alkaline and acid hydrolysis.

Conversion of alkylacyl-GPE to alkenylacyl-GPE

Alkylacyl-GPE, synthesized in reaction mixtures containing platelets and alkylacylglycerols, could be converted to alkenylacyl-GPE by incubation with fresh platelet homogenates (**Table 3**). Because the amount of alkylacyl-GPE in the mixture of ethanolamine phosphoglycerides used for this experiment was not determined, no conversion rate could be calculated.

Enzyme activity in platelet subcellular fractions

Platelets were disrupted by mechanical homogenization, and the constituents were separated by differential centrifugation or by sucrose-gradient centrifugation (11, 27). Whole homogenate, 1000 *g* supernate, 12,000 *g* supernate, 100,000 *g* supernate, resuspended 1000 *g* pellets, resuspended 12,000 *g* pellets, and resuspended 100,000 *g* pellets gave reaction rates of 0.24, 0.17, 0.08, 0.01, 0.26, 0.28, and 0.36 nmole/min/mg of platelet protein, respectively, when incubated for 30 min with 0.4–0.5 mg of protein per assay. There was a progressive loss of enzyme activity in the supernatant fractions and little increase in the activity of resuspended pellets. Under identical conditions, the reaction rates obtained with platelet membranes and granules were, respectively, 0.39 and 0.19 nmole/min/mg of protein.

Enzyme activity in blood cells

Reaction rates obtained with erythrocyte membranes, polymorphonuclear leukocytes, and lymphocytes were, respectively, 0.04, 0.02, and 0.1 nmole/min/mg of protein. Therefore, the small number of other blood cells present in the platelet concentrates did not affect the final results. The platelet enzyme separated from 10 normal subjects gave

reaction rates of 0.24–0.34 nmole/min/mg of protein. Similar reaction rates were found using platelet homogenates from 12 patients with coronary vascular disease, 2 patients with renal failure, and 1 patient with hepatic failure.

DISCUSSION

The enzyme cytidine-5′-diphosphate ethanolamine:1-radyl-2-acyl-*sn*-glycerol ethanolaminephosphotransferase was described by Kennedy (28) during his discovery of the involvement of the cytidine nucleotides in phosphoglyceride metabolism. Recent studies have characterized the properties of this enzyme from rat brain; in particular, the mammalian enzyme requires as substrate diradyl-*sn*-glycerols that have a 2-acyl constituent (29–31). There are as yet no data that would suggest that more than one enzyme is involved in the conversion of 1-radyl-2-acyl-*sn*-glycerols to ethanolamine phosphoglycerides. Our data demonstrate that platelets contain ethanolaminephosphotransferase and can thus produce ethanolamine phosphoglycerides. These data also suggest that the incorporation of radioactive glycerol and phosphate into the ethanolamine phosphoglycerides may represent, at least in part, de novo synthesis of phosphoglycerides (2, 3). The ethanolaminephosphotransferases of erythrocytes, lymphocytes, and polymorphonuclear leukocytes are substantially less active.

In other tissues, alkenylacyl-GPE is produced by the enzymatic oxidation of alkylacyl-GPE (32–35), and serine phosphoglycerides are produced by base exchange (7–10). Our preliminary data (Tables 1 and 3 and Fig. 6) suggest that both mechanisms are also operative in platelets. As performed, these experiments are not quantitative, and therefore the enzymatic activity cannot be calculated. The probable sequence of plasmalogen synthesis has been reviewed (32–34), and the characteristics of the 1-alkyl-2-acyl-glycero-3-phosphorylethanolamine desaturase from the mucosa of hamster small intestine have been investigated using chemically defined substrates (34). The exact manner of oxidation of alkylacyl-GPE to alkenylacyl-GPE remains to be determined but the reaction rates are increased by NADP in the presence of an NADPH-generating system (35). Because platelets and erythrocytes may contain the substrates CDP ethanolamine (17, 36) and diacylglycerols (37, 38) as well as the enzyme ethanolaminephosphotransferase, these cells may maintain their capacity to produce diacyl-GPE and may not be totally dependent on the exchange of fatty acids to prevent the depletion of these essential phosphoglycerides. It is not unexpected that platelets should contain appreciable amounts of the enzymes involved in phosphoglyceride synthesis because these lipids are necessary for their clot-promoting activity.

The ethanolaminephosphotransferase of platelets differs from the enzyme of rat brain microsomes in several ways. The apparent K_m of the platelet enzyme for CDP ethanolamine is 1.6×10^{-5} M as opposed to the value of $2.1–2.8 \times 10^{-4}$ M for the enzyme from rat brain. The affinity of the platelet enzyme for diacylglycerols is considerably greater than that determined using the enzyme from brain (29–31). Because the diacylglycerols in these in vitro experiments are present in the form of unstable emulsions, the calculated affinity of the enzyme for diacylglycerols does not reflect true enzyme–substrate equilibrium, which can be determined only at levels below the critical micelle concentration of diacylglycerols (39). In all preparations the pH optimum of the enzyme is 8.0–9.0, and Mn^{2+} is the preferred divalent cation. The platelet enzyme is activated by various bile salts and acids. This effect is more than can be explained by solubilization of the lipid substrate because other detergents are less active. Some detergents are inhibitory, as are low concentrations of monoolein and high concentrations of sodium glycodeoxycholate. In the absence of Mn^{2+} and CDP ethanolamine, the enzyme is rapidly inactivated by sodium glycodeoxycholate in the concentration (0.82 mM) used for these experiments. Both the activating and the inhibiting properties of bile salts are probably due to alterations of the membrane structure that affect the configuration of the enzyme. Whether the stimulation of ethanolaminephosphotransferase in platelets by bile salts and acids is unique to these cells remains to be determined.

Platelets incubated with radioactive acetate or glycerol in vitro produce ethanolamine phosphoglycerides at a rate of 0.2–1.0 pmole/min/mg of protein (40). If similar synthetic rates occur in vivo, platelet ethanolamine phosphoglycerides (80 nmoles/mg of protein) would be replaced very slowly (38). Our data demonstrate that ethanolaminephosphotransferase can synthesize 40–50 pmoles of ethanolamine phosphoglycerides/min/mg of protein when incubated with optimal concentrations of Mn^{2+} and CDP ethanolamine. The further addition of 1-radyl-2-acyl-*sn*-glycerols and sodium glycodeoxycholate increases the reaction rate to 0.24–0.34 nmole/min/mg of protein. It seems probable that ethanolaminephosphotransferase is inhibited in intact platelets either by the unavailability of substrates and cofactors or by other, undefined mechanisms. When assayed under optimal conditions, the activity of ethanolaminephosphotransferase was unchanged in platelets obtained from patients with renal failure or atherosclerosis of the coronary arteries. However, these data do not exclude a possible alteration of enzyme activity in vivo.

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