Diglyceride kinase in human platelets

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Abstract Human platelets contain diglyceride kinase, an enzyme that catalyzes the phosphorylation of diacylglycerol by adenosine 5′-triphosphate to yield phosphatidic acid. The majority of the platelet enzyme is particulate-bound, and membrane fractions of platelet homogenates have a higher specific activity than granule fractions. Both deoxycholate and magnesium are necessary for optimal enzyme activity. The K_m of the enzyme for adenosine 5′-triphosphate is 1.3 mM, and the apparent K_m for diacylglycerol is 0.4 mM. The pH optimum is 6.6–6.8 in imidazole–HCl or maleate–NaOH buffer. The enzyme activity of platelets from normal subjects was similar to the activity from patients with renal and hepatic failure.

Supplementary key words 1,2-diacylglycerol · diolein · monoolein · adenosine 5′-triphosphate · phosphatidic acid · deoxycholate · erythrocyte membranes · lymphocytes

A NUMBER OF laboratories have demonstrated that platelets incorporate radioactive phosphate into phosphoglycerides in vitro (1–6). Phosphatidic acid and phosphatidylinositol are labeled more intensely than other phosphoglycerides, although they represent only a small proportion of the total phosphoglycerides. The labeling of phosphatidic acid and phosphatidylinositol is increased when platelets are incubated in hypotonic buffer or exposed to adenosine 5′-diphosphate (6, 7). These in vitro manipulations are associated with changes in platelet volume and shape that also occur during the initial phases of platelet aggregation.

The enzymatic mechanisms of synthesis of phosphatidic acid in these situations are unknown. Platelets are able to produce phosphatidic acid from D,L-α-glycerophosphate and acyl coenzyme A (8). However, platelet studies in which radioactive acetate and palmitate are utilized show little incorporation of the isotope into phosphatidic acid (9, 10). Furthermore, when platelets are incubated with radioactive glycerol, the isotope is well distributed among the various phospholipids (8). These data suggest that the incorporation of radioactive phosphate into phosphatidic acid in platelets represents a phosphate exchange rather than de novo synthesis. Hokin and coworkers (11–17) have examined the selective incorporation of radioactive phosphate into phosphatidic acid and phosphatidylinositol of neural, exocrine, and endocrine tissues. They have demonstrated the enzyme diglyceride kinase (adenosine triphosphate: diglyceride phosphotransferase) and have accrued evidence that this enzyme is involved in the incorporation of radioactive phosphate into phosphoglycerides. The present work demonstrates that human platelets contain diglyceride kinase, and the characteristics of the enzyme are examined. Since the enzyme may be active in the early phases of platelet aggregation, we have investigated the enzyme in platelets that aggregate poorly (obtained from patients with renal and hepatic failure). A preliminary report of this work has been published (18).

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; CTAB, cetyltrimethylammonium bromide.

METHODS

Preparation of cell homogenates

Platelets, erythrocyte membranes, lymphocytes, and polymorphonuclear leukocytes were separated from fresh whole blood as previously described (19). Cells were resuspended in 0.25 M sucrose containing 1 mM disodium ethylenediaminetetraacetate (Na_2EDTA), pH 7.0, and 0.01 M 2-mercaptoethanol. Unless indicated, the cells were disrupted by sonication using a Biosonik III instrument with a microtip and an intensity setting of 60 (Bronwill Scientific, Inc., Rochester, N.Y.). The protein concentration was adjusted to 1.0–1.5 mg/ml.

Preparation of radioactive adenosine 5′-triphosphate (ATP)

[γ-^{32}P]ATP was prepared from [^{32}P]phosphoric acid (New England Nuclear, Boston, Mass.) as described by Glynn and Chappell (20) and was purified by Dowex-1-Cl chromatography. Paper chromatography, using 1 M

ammonium acetate, 0.01 M Na_2EDTA (pH 7.5)–90% ethanol 6:14 (v/v) (descending) and isobutyric acid–0.1 M Na_2EDTA–H_2O–NH_4OH 100:1.6:55.8:4.2 (by vol) (ascending), demonstrated one radioactive spot that migrated in the same position as ATP (21). Prior to each experiment, Na_2ATP (Sigma grade, Sigma Chemical Co., St. Louis, Mo.), neutralized by the addition of NaOH, was added to the radioactive ATP so that the final specific activity was 3.2–6.4 × 10^8 cpm/mmole.

Diacylglycerol

1,2-Diacylglycerol was prepared from egg phosphatidylcholine by a modification of the procedure described by Hanahan and Vercamer (22). 1 ml of 40 mM $CaCl_2$ and 25 mg of phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3), dissolved in 5 ml of 0.05 M imidazole–HCl buffer (pH 7.1), were added in sequence to 1.5 mmoles of phosphatidylcholine (dissolved in 100 ml of diethyl ether–ethanol 100:2 [v/v]). The mixture was left at room temperature, and aliquots were removed periodically to determine by thin-layer chromatography the extent of production of diacylglycerol. After 120 min, the ether phase was washed with water and dried over anhydrous Na_2SO_4. The 1,2-diacylglycerol was purified by silicic acid chromatography (23). The lipid eluted with *n*-hexane–diethyl ether 7/3 (v/v) had an ester to glycerol ratio of 1.96:1.0. Thin-layer chromatography on silica gel containing $CaSO_4$, using diethyl ether–benzene–ethanol–acetic acid 40:50:2:0.2 (by vol) and petroleum ether–diethyl ether–acetic acid 80:20:1 (v/v/v) showed single spots with R_F values of 0.58 and 0.17, respectively (24, 25). No 1,3-diacylglycerol was detected. The yield of 1,2-diacylglycerol was 83–92%.

Other reagents

Phosphatidic acid, lysophosphatidic acid, and phosphatidylcholine were prepared as previously described (19). Diolein, monoolein, adenosine 5′-diphosphate (ADP, Sigma grade 1, sodium salt), and D,L-α-glycerophosphate were obtained from Sigma.

Other methods

Protein and lipid esters were determined as previously described (19). Lipid glycerol was determined by the method of Hanahan and Olley (26). Phospholipids were subjected to mild alkaline hydrolysis, and the deacylated, water-soluble phosphates were separated by paper chromatography or by Dowex-1–formate column chromatography using a linear gradient of 2 N formic acid (27–29). Silicic acid chromatography was performed in 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 emulsions were prepared by sonication of mixtures of lipid, detergent, and buffer salts immediately prior to each experiment, using the conditions employed for cell disruption (see above). Unless indicated, other reagents were added directly to the reaction mixtures and were not present during the preparation of the emulsions.

Enzyme assay

The standard assay mixture contained 5 mM [γ-^{32}P]-ATP, 0.48 mg/ml deoxycholate, 0.96 mM diacylglycerol, 40 mM imidazole–HCl (pH 6.6), 0.1 M sucrose, 0.4 mM Na_2EDTA, 4 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and enzyme in a final volume of 0.5 ml. The mixtures were incubated at 30°C under air in 50-ml glass-stoppered centrifuge tubes. The reactions were stopped by the addition of 5 ml of methanolic 0.1 N HCl. Chloroform, 10 ml, (containing 0.1 μmole of phosphatidic acid) was added, and the chloroform extract was washed three times with 20 ml of 2 M KCl. An aliquot of the chloroform phase was dried, and the radioactivity was determined with a Packard liquid scintillation counter (19). The quantity of phosphatidic acid synthesized was calculated by dividing the radioactivity detected by the specific activity of the [γ-^{32}P]ATP.

RESULTS

Initial experiments

In the presence of [γ-^{32}P]ATP, platelet suspensions incorporated very small amounts of radioactive phosphate into a chloroform-soluble product (3 pmoles/min/mg protein). The presence of deoxycholate, 0.4 mg/ml, and 20 mM $MgCl_2$ resulted in an increase of chloroform-soluble radioactive phosphate to 50 pmoles/min/mg protein. Neither deoxycholate nor $MgCl_2$ was effective alone. The addition of 0.1 mM diacylglycerol in the presence of deoxycholate and $MgCl_2$ resulted in a marked increase in the chloroform-soluble radioactive phosphate (0.7 nmoles/min/mg protein). Platelets disrupted by sonication or freezing and thawing were three to four times more effective than intact platelets in this regard. Unless indicated, platelets disrupted by sonication were used in the remainder of the studies.

Shaking the tubes during the incubation period did not affect the reaction rate. The enzyme activity was greatest when the platelet homogenates were added last to the combination of reagents, and all subsequent experiments were performed in this manner.

Product identification

The reaction mixture was identical with that outlined in Fig. 1, except the [γ-^{32}P]ATP had a sp act of 16 × 10^8 cpm/mmole. The reaction was stopped and extracted as

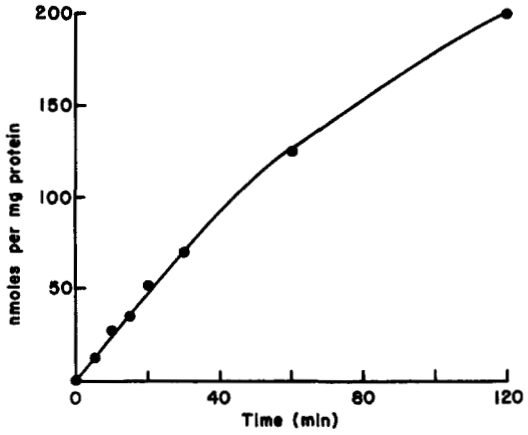

FIG. 1. Production of phosphatidic acid at different time intervals. The reaction mixture contained 0.5 mg of protein derived from platelets disrupted by sonication, 0.1 M sucrose, 0.4 mM Na_2EDTA, 4 mM 2-mercaptoethanol, 0.48 mg/ml deoxycholate, 0.96 mM diacylglycerol, 5 mM Na [γ-^{32}P]ATP, 10 mM $MgCl_2$, and 40 mM imidazole–HCl in a total volume of 0.5 ml at a final pH of 6.6. The reaction mixture was incubated at 30°C for the time indicated.

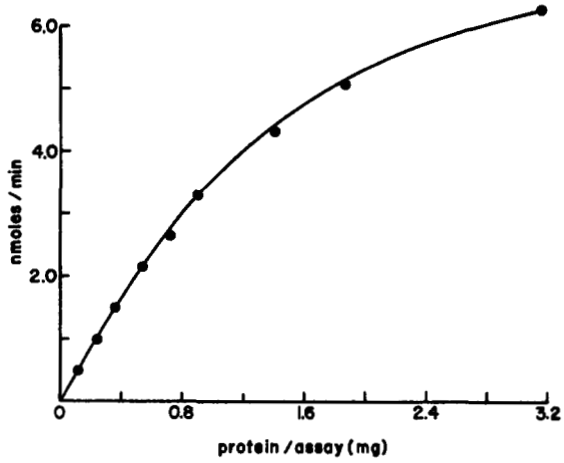

FIG. 2. Production of phosphatidic acid at different protein concentrations. Conditions were identical with those described in Fig. 1 except the reaction mixtures were incubated for 20 min and contained platelet protein in the amount indicated.

indicated above except that 10 μmoles of Tris phosphatidate was added. After the washes with 2 M KCl, aliquots of the chloroform phase were studied by chromatography and alkaline hydrolysis. 97.1% of the radioactivity migrated with phosphatidic acid on thin-layer silica plates developed with chloroform–pyridine–formic acid 50:20:7 (v/v) (R_F 0.59) (19). On silicic acid column chromatograpy, 94% of the radioactive product was eluted in a sharp peak with chloroform–methanol 94:6 (v/v) (19). When the product was deacylated by mild alkaline hydrolysis, 96.2% of the radioactivity was recovered in the aqueous extracts. The deacylated product migrated in the same position as α-glycerophosphate, as judged by ascending paper chromatography using as solvents methanol–formic acid–water 80:13:7 (v/v/v) and *n*-propanol–NH_4OH–water 5:4:1 (v/v/v) (R_F 0.67 and 0.54, respectively). The identity of the deacylated radioactive product was confirmed by superimposition of the radioactivity on the α-glycerophosphate peak during Dowex-1–formate column chromatography.

Time course

The reaction was linear in respect to time for 20 min (Fig. 1). The reaction continued thereafter but the rate at 120 min was 40% of the original rate. At 120 min, 4% of the ATP and 21% of the diacylglycerol had been utilized for the production of phosphatidic acid.

Enzyme concentration

The reaction rate was linear with respect to the concentration of platelet protein up to 0.36 mg/assay or 0.72 mg/ml (Fig. 2). The reaction rate decreased from 4.2 to 2.0 nmoles/min/mg protein when the protein concentration was increased from 0.72 to 6.3 mg/ml.

pH optimum and effect of buffer salts

The enzyme was active over a broad pH range of 6.0 to 8.6. In imidazole–HCl or maleate–NaOH, the pH optimum was 6.6–6.8 (Fig. 3). The activity was unchanged when the concentration of imidazole–HCl, pH 6.6, was raised from 10 to 40 mM, but a 37 and 64% inhibition occurred at 60 and 80 mM, respectively. An increase in the concentration of Tris–HCl buffer (pH 7.4) from 40 mM to 0.2 M resulted in a 36% inhibition. The pH optimum was 7.4 in 40 mM potassium phosphate buffer, and the entire curve was shifted towards a higher pH. Thus, phosphate buffer was a more satisfactory buffer at pH 7.4–8.0 but caused enzyme inhibition at pH 6.2–7.0.

Substrates and cofactors

Table 1 shows the effect of omission of some of the reagents from the complete reaction mixture. Small amounts of chloroform-soluble radioactive phosphate were produced in the absence of diacylglycerol. The affinity of the enzyme for diacylglycerol is illustrated in Fig. 4. In this experiment the ratio of diacylglycerol to deoxycholate was maintained at 2 μmoles to 1 mg. The apparent K_m of the enzyme for diacylglycerol was 0.4 mM when calculated from a Lineweaver-Burk plot of the reciprocal of the reaction rate vs. the reciprocal of the substrate concentration. The reaction rate using 0.96 mM diacylglycerol was approximately 93% of that achieved using diacylglycerol concentrations up to 5 mM. When the concentration of deoxycholate was maintained at 0.48 mg/ml, a diacylglycerol concentration of 0.375 mM produced a reaction rate that was 85% of that achieved at 5 mM (not illustrated). The initial portion

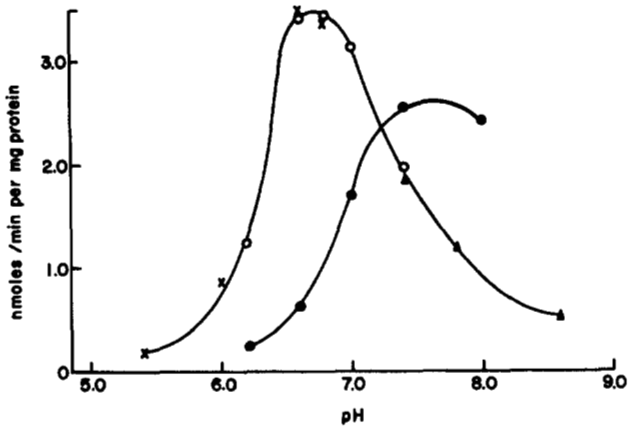

FIG. 3. Effects of pH and buffer salts on phosphatidic acid production. Conditions were identical with those described in Fig. 1 except the reaction mixtures contained 0.61 mg of platelet protein and were incubated for 20 min. The pH and buffer salt were as indicated but the final buffer salt concentration was 40 mM. ×, maleate–NaOH; ○, imidazole–HCl; ▲, Tris–HCl; ●, potassium phosphate.

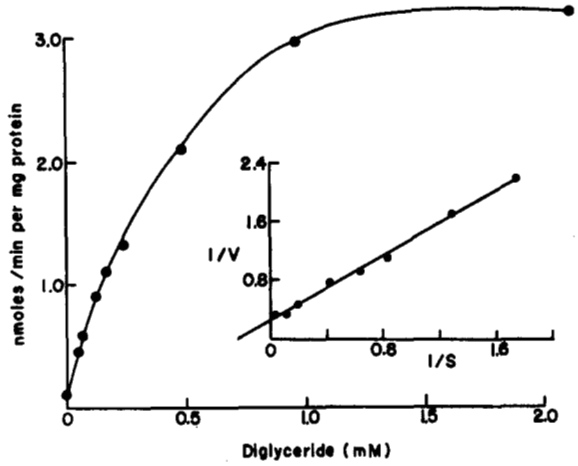

FIG. 4. Production of phosphatidic acid at different concentrations of diacylglycerol. Conditions were identical with those detailed in Fig. 1 except the concentration of diacylglycerol was as indicated and the time of incubation was 20 min. The ratio of diacylglycerol to deoxycholate was maintained at 2 μmoles to 1 mg.

of the curve of reaction rate vs. substrate concentration became sigmoidal and a K_m could not be calculated by using a Lineweaver-Burk plot. The reaction rate was unchanged when 0.96 mM diolein was substituted for the mixture of diacylglycerols.

When 0.96 mM monoolein was substituted for diacylglycerol, the reaction rate was only 12–16% of that obtained with diacylglycerol. When the products of this reaction were studied by thin-layer chromatography (19), 18–27% of the radioactivity migrated in the same position as lysophosphatidic acid, both on plates of silica gel containing Na_2CO_3 developed with chloroform–methanol–acetic acid–water 25:15:4:2 (by vol) (R_F 0.47) and on plates of silica gel developed with chloroform–pyridine–formic acid 50:20:7 (v/v/v) (R_F 0.23). The remainder of the radioactivity migrated as phosphatidic acid (R_F 0.9 and 0.59, respectively). After mild alkaline hydrolysis of the reaction products, 98% of the radioactivity was recovered in the aqueous extract, and the deacylated product migrated in the same position as α-glycerophosphate during paper chromatography using two solvent systems (see product identification section).

Calculated from a Lineweaver-Burk plot, the K_m of the enzyme for ATP was 1.3 mM when the diacylglycerol concentration was 0.96 mM (Fig. 5). The K_m appears to be 0.75 mM when estimated by inspection of the plot of the reaction rate vs. the substrate concentration. The reaction rates were not determined using a fixed ATP to magnesium ratio.

Magnesium was necessary for enzyme activity (Table 1). The optimal reaction rate was obtained using 10 mM $MgCl_2$. A further increase in $MgCl_2$ concentration to 20 and 40 mM decreased the reaction rate by 11 and 55%, respectively. These higher concentrations of $MgCl_2$ also decreased the reaction rate in experiments using potassium phosphate buffers at pH 6.7 and 7.4. 10 mM $CaCl_2$ and 10 mM $MnCl_2$ were 33 and 17%, respectively, as effective as 10 mM $MgCl_2$. No enzyme activity occurred in the presence of 10 mM $CoCl_2$, $ZnSO_4$, $FeSO_4$, or $CuSO_4$.

Effect of thiol reagents and sulfhydryl-binding agents

The rates of synthesis of phosphatidic acid by platelet homogenates containing thiol reagents were compared with those obtained using homogenates alone. When 4 to 40 mM 2-mercaptoethanol was added, the reaction rate was increased by 6%. The reaction rate was not affected by 2 mM 2-mercaptoethanol, and 80 mM 2-mercaptoethanol decreased the reaction rate by 11%. 6.4 mM dithiothreitol increased the reaction rate by 6%,

TABLE 1. Effect of omission of reagents from complete reaction mixture

Omission from Reaction Mixture	Phosphatidic Acid Produced
	nmoles/min/mg protein
None	3.8
2-Mercaptoethanol	3.6
Na_2EDTA	3.8
Diacylglycerol	0.08
Deoxycholate	0.04
Mg^{2+}	0
Enzyme	0

0.5 mg of platelet protein was present except where indicated, and the time of incubation was 20 min; otherwise, the complete reaction mixture was identical with that detailed in Fig. 1.

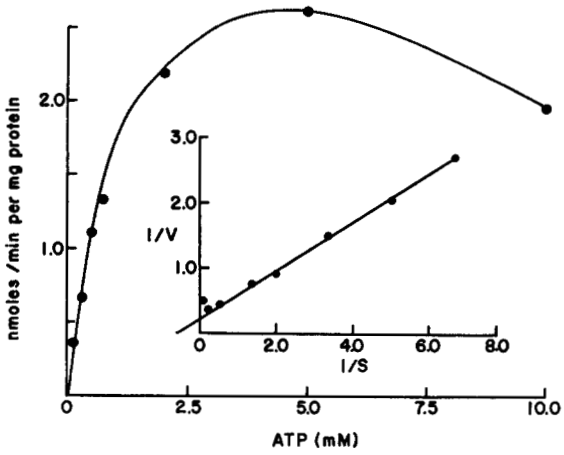

FIG. 5. Production of phosphatidic acid at different concentrations of [γ-^{32}P]ATP. Conditions were identical with those detailed in Fig. 1 except the time of incubation was 20 min and the concentration of [γ-^{32}P]ATP was as indicated (sp act, 3.2×10^8 cpm/mmole when the ATP concentrations were 0.75–10.0 mM and 32×10^8 cpm/mmole when the ATP concentrations were 0.15–0.5 mM).

and reduced glutathione decreased the reaction rate by 26%. Parachloromercuribenzoate, 0.1 mM, inactivated the enzyme but 6.4 mM dithiothreitol, 6.4 mM reduced glutathione, and 20 mM 2-mercaptoethanol restored 60, 43, and 20% of the original activity, respectively. 2 and 20 mM sodium iodoacetate inhibited the enzyme by 17 and 57%, respectively, and the subsequent addition of dithiothreitol or 2-mercaptoethanol did not reverse the inhibition.

Temperature effects

The reaction rates at various temperatures were compared with the reaction rate at 37°C (Table 2). The reaction rate was greatest at 30°C, and, unless indicated, all experiments were performed at this temperature. Platelet homogenates that were boiled for 5 min were unable to produce phosphatidic acid and were routinely used as controls for all experiments. Platelets disrupted by sonication and stored for 10 days at 4°C and for 35 days at −20°C lost 35 and 20% of their initial activity, respectively. Platelet homogenates incubated at 30°C for 30 and 60 min prior to the assay of enzyme activity lost 8 and 39% of their initial activity, respectively (Table 3). In the studies reported here, the platelet homogenates were used immediately following preparation or were stored at −20°C for no longer than 10 days.

Heat inactivation studies were performed to investigate the possibility that isoenzymes might be responsible for the changes in enzyme activity in imidazole and phosphate buffers. In one experiment, platelets were incubated at 50°C for 10 min and then assayed in 40 mM potassium phosphate (pH 6.6 and 7.4) or imidazole–HCl (pH 6.6 and 7.4). The amount of phosphatidic acid

TABLE 2. Effect of temperature on the rate of phosphatidic acid synthesis

Temperature of Incubation	Phosphatidic Acid Produced
	nmoles/min/mg protein
4°C	0.45
21°C	3.45
30°C	3.95
37°C	3.3
50°C	0.35
65°C	0.05

The conditions of the experiment were identical with those detailed in Fig. 1. except that the reaction mixture contained 0.48 mg of protein and the mixture was incubated for 20 min at the temperature indicated.

synthesized by these preparations was 1.6–2.5% of that synthesized by untreated homogenates assayed under identical conditions.

Effects of sodium deoxycholate and other detergents

Solutions of diacylglycerol prepared from egg phosphatidylcholine and buffer salts did not form emulsions when sonicated. Addition of detergents allowed the preparation of emulsions that were stable at room temperature, generally for several hours. Sodium deoxycholate increased the production of phosphatidic acid by platelet homogenates, even when diacylglycerol was not present (see Initial Experiments, above). Emulsions of diacylglycerol prepared with sodium deoxycholate produced a marked increase in the reaction rate (Table 1). When 2 mM diacylglycerol was used, the optimal reaction rate occurred at deoxycholate concentrations of 0.48–0.8 mg/ml. When the diacylglycerol concentration was decreased to 0.96 mM, optimal reaction rates occurred with a deoxycholate concentration as low as 0.32 mg/ml.

Although deoxycholate stimulated the production of phosphatidic acid by platelet homogenates and formed the emulsions of diacylglycerol that were necessary to demonstrate the maximal effect of this substrate, difficulties in its use were evident. Because of the poor solubility of deoxycholic acid in aqueous solutions and because of the relatively high pK_a of dilute solutions of deoxycholic acid (5.17), precipitation of deoxycholic acid occurred at pH 6.6–6.8 before the concentration of sodium deoxycholate could be increased above the critical micellar concentration (30, 31). Using a diacylglycerol concentration of 0.96 mM, visible flocculation developed in the emulsions containing 0.64–1.28 mg of deoxycholate/ml. When these unstable emulsions were used immediately after sonication, the usual reaction rate occurred. Secondly, in the absence of $MgCl_2$ and

TABLE 3. Effect of preincubation of platelet homogenates on production of phosphatidic acid

Preincubation	Addition to Reaction Mixture	Phosphatidic Acid Produced
		nmoles/min/mg protein
None	None	3.7
30 min	None	3.4
60 min	None	2.3
30 min, emulsion	None	1.0
30 min, emulsion + PA	PA	2.0
30 min, emulsion + PC	PC	2.0
None	PA	4.6
None	PC	4.9

0.48 mg of platelet protein was preincubated at 30°C under air with or without lipid emulsions. During preincubation, emulsions contained 50 mM imidazole–HCl (pH 6.6), 0.6 mg/ml sodium deoxycholate, and 1.2 mM diacylglycerol with addition of 0.25 mM phosphatidic acid (PA) or 0.25 mM phosphatidylcholine (PC) as indicated. The final reaction mixture was identical with that detailed in Fig. 1, except that phosphatidic acid and phosphatidylcholine in a final concentration of 0.2 mM were present as indicated. The time of incubation was 20 min.

TABLE 4. Effects of detergents on the production of phosphatidic acid

Detergent Used to Prepare Emulsion	Detergent Added	Phosphatidic Acid Produced
		nmoles/min/mg protein
Deoxycholate	None	3.4
Deoxycholate	Cutscum	0.58
Deoxycholate	Triton X-100	0.58
Deoxycholate	CTAB	1.53
Cutscum	None	0
Cutscum	Deoxycholate	2.42
Cholate	None	1.35
CTAB	None	0.67

Emulsions were prepared with detergents (1.2 mg/ml) so that the final concentration of detergent in the incubation mixture was 0.48 mg/ml. Prior to incubation, 0.03 ml of another detergent (8 mg/ml) was added where indicated. The time of incubation was 20 min and the amount of platelet protein was 0.52 mg; otherwise, the reaction mixture was identical with that detailed in Fig. 1.

Na_2ATP, emulsions of sodium deoxycholate and diacylglycerol increased the loss of enzyme activity that occurred when platelet homogenates were preincubated at 30°C (Table 3).

Control experiments demonstrated that deoxycholate had no effect on the extraction and washing procedures. Thus, when the deoxycholate concentration was varied from 0 to 4 mg/ml, all of the [γ-^{32}P]ATP was removed from the chloroform phase by the 2 M KCl washes, and 98–101% of 1 μmole of added phosphatidic acid remained in the chloroform phase.

Attempts to use other detergents were generally unsuccessful. Sodium cholate and cetyltrimethylammonium bromide (CTAB), both 0.48 mg/ml, were, respectively, 40 and 20% as effective as deoxycholate (Table 4). Cutscum (isooctylphenoxypolyoxyethylene), Triton X-100 (octylphenoxypolyethoxyethanol), Tween 80 (polyoxyethylene sorbitan monooleate), and sodium alkyl sulfates could not be substituted for deoxycholate. When emulsions of diacylglycerol were prepared with Cutscum, the addition of sodium deoxycholate in a final concentration of 0.48 mg/ml increased the reaction rate to 71–76% of that obtained using emulsions prepared with deoxycholate (Table 4). On the other hand, the addition of Cutscum, Triton X-100, or CTAB, all in a final concentration of 0.48 mg/ml, to the complete reaction mixture (containing sodium deoxycholate) decreased the reaction rate by 83, 83, and 55%, respectively (Table 4).

Reaction products

The effects of the reaction products on the enzyme activity were studied by their addition to the reaction mixture. ADP, 1 mM, decreased the reaction rate by 32%. Kinetic analysis indicated that ADP functioned as a competitive inhibitor of ATP. Inhibition by phosphatidic acid could not be demonstrated. Instead, the addition of Tris phosphatidate, 0.1, 0.2, and 0.3 mM, increased the apparent production of radioactive phosphatidic acid by 21, 29, and 33%, respectively. When phosphatidic acid was added during the preparation of emulsions, a similar effect was noted (Table 3). Moreover, the presence of phosphatidic acid during the preincubation of platelet homogenates with deoxycholate and diacylglycerol reduced the loss of enzyme activity (Table 3).

To establish whether this effect was specific for phosphatidic acid, another phosphoglyceride was investigated. When phosphatidylcholine, 0.1 and 0.2 mM, was added to the reaction mixture, the reaction rate was increased by only 5 and 6%, respectively. On the other hand, when phosphatidylcholine was added during the preparation of emulsions, it effected an increase in the reaction rate similar to that of phosphatidic acid (Table 3).

Role of phosphatidate phosphohydrolase

Platelets have been shown to contain phosphatidate phosphohydrolase (18). The apparent stimulation of the enzyme by phosphatidic acid could have occurred because this enzyme hydrolyzed the phosphatidic acid to diacylglycerol, thus increasing substrate concentration. Alternatively, if phosphatidate phosphohydrolase were active in the reaction mixtures, the addition of unlabeled phosphatidic acid could protect the labeled phosphatidic acid from degradation, and thus appear to be stimulating the reaction. Sodium fluoride is an inhibitor of phosphatidate phosphohydrolase (32), and its effect was

studied. 4–16 mM sodium fluoride increased the production of phosphatidic acid by approximately 10%. 24 mM sodium fluoride had no effect, and 32 mM sodium fluoride decreased the production by 5%. Furthermore, when radioactive phosphatidic acid (separated by solvent extraction from standard reaction mixtures) was incubated with platelet homogenates under the conditions of these experiments for 20 min, only 4% of the radioactive phosphatidic acid disappeared. This disappearance was eliminated by the addition of 16 mM sodium fluoride.

Effect of salt concentration

Because of the reduction of enzyme activity by higher concentrations of buffer salts, other salts or osmotically active agents were added to the complete reaction mixture to determine their effects on the enzyme. However, when added in this manner, albumin, 0.15 and 0.3 mg/ml, 40 mM NH_4Cl, 40 mM KCl, 100 mM NaCl, and 150 mM sorbitol did not alter the reaction rate.

Enzyme activity in platelet subcellular fractions

Platelets were homogenized with a Teflon pestle, and fractions were separated by centrifugation at 1000, 12,000, and 100,000 *g* (19). The 100,000 *g* supernatant fraction and each of the resuspended pellets had the same specific activity as the original homogenate. Platelet membranes and granules were separated by centrifugation on sucrose gradients (19). The enzyme activity of platelet membranes was similar to that of the original homogenate. The enzyme activity of the granules was 50% of the activity of the other platelet fractions.

Attempts at enzyme isolation

Freezing and thawing, mechanical homogenization, and sonication of platelets in 0.25 M sucrose followed by centrifugation at 100,000 *g* for 60 min released 10, 34, and 48%, respectively, of the enzyme activity into the supernatant with no overall loss of activity. Precipitation of the supernatant with acetone at −20°C and with methanol–water 2:1 (v/v) at 4°C caused a 80–90% loss in enzyme activity (19). Dialysis of the supernatant fraction against 0.15 M NaCl–0.02 M imidazole (pH 6.6) for 24 hr at 4°C resulted in a 93–98% loss of activity. Precipitation with 50% NH_4SO_4 or gel exclusion chromatography on Bio-Gel A-5m completely inactivated the enzyme. Sonication of platelets suspended in 5 mM Tris–HCl (pH 7.0), 0.35 M NaCl, or 5 mg/ml deoxycholate resulted in 63, 69, and 96% loss of activity, respectively.

Enzyme activity of other blood cells

The reaction rates for erythrocyte membranes and lymphocytes were 2.05 and 6.37 nmoles/min/mg protein, respectively. No enzyme activity was demonstrated in polymorphonuclear neutrophils. Since the platelet preparations were relatively free from contaminating cells (19), less than 1% of the observed activity could be explained by the activity of other cells.

Enzyme activity in the platelets of normal subjects and patients

The reaction rate in platelet homogenates from 12 normal subjects was 3.86–4.71 nmoles/min/mg protein. The reaction rates in platelet homogenates from eight patients with renal failure (blood urea nitrogen greater than 90 mg per 100 ml) and from six patients with hepatic failure (bilirubin greater than 10 mg per 100 ml) were similar to normal values.

DISCUSSION

Hokin and Hokin (11–15), Hokin (16), and Sastry and Hokin (17) have described the enzyme diglyceride kinase, and it has been shown (13, 15) in salt gland and pancreas that the phosphatidic acid synthesized by this enzyme can be converted subsequently to phosphatidylinositol. These investigators have presented a number of observations that support the possibility that this enzyme is involved in the incorporation of radioactive phosphate into phosphoglycerides that occurs during acetylcholine stimulation of neural cortex, salt gland, and pancreas, or during phagocytosis by peritoneal leukocytes (12, 14, 16, 17). The demonstration of diglyceride kinase in platelets raises the possibility that this enzyme might be important in platelet function. Activation of this enzyme would result in the high rate of incorporation of radioactive phosphate into phosphatidic acid and phosphatidylinositol that is observed in platelets studied in vitro.

The activity of the enzyme in human platelets expressed as nmoles/min/mg protein is 3.9–4.7 units, a value similar to the activity of *Escherichia coli* (3–4 units) (33, 34). The activity in platelets is somewhat greater than that of guinea pig pancreas homogenates (1.22 units), human erythrocyte membranes (0.5 or 2.1 units), and phagocytic leukocytes (0.57 units) but is less than mononuclear leukocytes (6.4 units) (17, 35, 36). No extensive investigation of the intracellular localization has been reported for any of these cells, but the majority of the enzyme is particulate-bound. In homogenates of the albatross salt gland, the microsomal fraction is 4 times more active than the supernatant and mitochondrial fractions and is 1.5 times more active than the nuclear and cell debris fraction (13). In platelets disrupted by freezing and thawing, 90% of the enzyme remains in the 100,000 *g* sediment, and the intracellular fractions except for the granules have the same activity as the entire homogenate.

The platelet enzyme has a pH optimum of 6.6–6.8. The rapid loss of activity in potassium phosphate buffer below pH 7.4 is in some part due to the instability of the deoxycholate–diacylglycerol emulsions under these conditions. The enzyme of other tissues has been assayed from pH 6.5 to 7.4. The pH optimum in *E. coli* is 7.0 (33).

The enzyme in platelets is inhibited by high buffer salt concentrations of Tris and imidazole. In the presence of deoxycholate, low concentrations of K^+, Na^+, NH_4^+, and sorbitol did not change the enzyme activity. The enzyme activity in erythrocyte membranes is increased by Na^+ and K^+ in the presence but not in the absence of diacylglycerol (36). This effect may be related to the ability of cations to change the surface tension of lipid emulsions (37). The enzyme in *E. coli* has an ionic strength requirement that is provided for by either Mg^{2+} or NH_4^+ (33). In all studies of diglyceride kinase, divalent cations have been necessary for optimal enzyme activity. Mg^{2+} is most effective, but in platelets, Ca^{2+} can substitute partially.

In most studies, detergents have been necessary to demonstrate optimal activity of diglyceride kinase. Their effect is complex. First, detergents may alter the membrane containing the enzyme so that the incorporation of radioactivity from [γ-^{32}P]ATP into phosphatidic acid is increased. In guinea pig brain microsomes this occurs at low deoxycholate concentrations and is similar to the effect produced by acetylcholine and by incubation in hypotonic buffer (12). In the presence of Mg^{2+}, deoxycholate stimulates the incorporation of radioactivity of [γ-^{32}P]ATP into the chloroform extract of platelets both in the presence and absence of diacylglycerol. Second, detergents improve the emulsification of diacylglycerol and therefore increase substrate availability. This appears to be the major effect of deoxycholate in the present assay system. In two studies diacylglycerol emulsions have been successfully prepared with sonication alone, and the enzyme–substrate kinetics resemble those obtained with platelet (35, 36). Third, detergents inhibit the platelet enzymes that utilize phosphatidic acid and may thus increase product recovery (18, 19). On the other hand, the diglyceride kinase in erythrocyte membranes, assayed with unemulsified diacylglycerol, is inhibited by deoxycholate (36). In the absence of ATP, deoxycholate accelerates the inactivation of the enzyme in platelet homogenates incubated at 30°C.

The ability of phosphatidic acid to increase the recovery of radioactive phosphatidic acid in this assay system is unexplained. Phosphatidate phosphohydrolase is inhibited, to a large degree, by the conditions of these experiments, and thus the activity of this enzyme could explain only a fraction of this apparent stimulation. Phosphatidylcholine has a similar effect when it is added during the preparation of the lipid emulsions but a less marked effect when added directly to the reaction mixtures. These phospholipids may protect the enzyme from inhibition by deoxycholate.

The selective labeling of phosphatidic acid and its derivatives with radioactive phosphate during in vitro incubations may be explained in part by diglyceride kinase. An alternate explanation, which relies on the inability of radioactive phosphate to be incorporated into the phosphorylated precursors of phosphatidylcholine and phosphatidylethanolamine, is less likely (6). Holmsen (38) has demonstrated the labeling of phosphorylcholine, phosphorylethanolamine, and cytidine diphosphocholine in platelets incubated with radioactive phosphate. We have confirmed his finding using platelets and radioactive phosphate as well as platelet homogenates and [γ-^{32}P]ATP.

Whether the accelerated labeling of phosphoglycerides with radioactive phosphate is related to an alteration of the membrane of physiological significance remains to be determined. An alteration of the membrane could result in the release of diacylglycerols from the hydrolysis of phosphatidylinositols (39). If this were so, diglyceride kinase could save a portion of the lipid and initiate the reconstruction of the entire molecule. Exposure of platelets to thrombin also causes major changes in the labeling of phosphoglycerides with radioactive glycerol (8). Since platelets have hemostatic and coagulant functions that may be related to their phosphoglycerides, these phenomena require further study.

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