Lipid metabolism in human platelets: *II. De novo phospholipid synthesis and the effect of thrombin on the pattern of synthesis*

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Lipid Metabolism in Human Platelets

II. *DE NOVO* PHOSPHOLIPID SYNTHESIS AND THE EFFECT OF THROMBIN ON THE PATTERN OF SYNTHESIS

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ABSTRACT Washed human platelets were incubated with radioactive glycerol; the platelets were able to synthesize *de novo* the major phosphoglycerides including phosphatidic acid, phosphatidylinositol, phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine. The specific activities of the phosphoglycerides obtained after glycerol incorporation indicate that phosphatidic acid, phosphatidylinositol, and phosphatidyl choline are metabolically active relative to phosphatidyl ethanolamine and that formation of phosphatidyl serine occurs to a much more limited extent. When platelets were incubated with bovine thrombin, 1 U/ml, the pattern of glycerol incorporation into phospholipid was changed. There was a 3-fold decrease in the total incorporation into lipid in 30 min with a relative 5-fold decreased incorporation into phosphatidyl choline and phosphatidyl ethanolamine and a 5-fold increased incorporation into phosphatidyl serine. The increased incorporation into phosphatidyl serine was maximal within the first 2 min but was transient, since within 20 minutes, the rate returned to that seen in platelets incubated with glycerol alone. Purified human thrombin also produced this same effect on phospholipid synthesis in platelets. Trypsin produced effects on phosphoglyceride formation similar to those seen with thrombin, and the trypsin-induced effect was inhibited by prior incubation of trypsin with soybean trypsin inhibitor, suggesting that proteolysis may be required for the observed effects on phospholipid synthesis.

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INTRODUCTION

Neither mature leukocytes nor erythrocytes from human blood are capable of *de novo* fatty acid synthesis because they lack acetyl-coenzyme A (CoA) carboxylase, the first enzyme in this pathway (1, 2). Human platelets contain acetyl-CoA carboxylase (3), as well as fatty acid synthetase, and are thus able to synthesize fatty acids de novo (3–5). Because of this finding, we have now studied human platelets for their ability to synthesize phospholipids *de novo*. Previous studies have demonstrated that platelets incorporate phosphate into phospholipid (6) but have not distinguished between *de novo* synthesis and exchange of phosphate into preformed lipid. Recent studies using platelet extracts suggest that human platelets contain enzymes capable of synthesizing phosphatidic acid (3) and phosphatidylinositol (7) *de novo*.

In the present study, we have incubated washed intact platelets with radioactive glycerol and measured the incorporation of this compound into phosphoglycerides. The ability of this technique to demonstrate phospholipid synthesis depends on the presence in platelets of the enzyme glycerokinase, which converts glycerol to sn-glycerol 3-phosphate.[1] The sn-glycerol 3-phosphate thus formed may then be converted to phosphatidic acid, which is the precursor of the other phosphoglycerides (8, 9). Since no mechanism has been demonstrated for exchange of the glycerol backbone of phospholipids, any incorporation of glycerol is assumed to reflect *de novo* synthesis. In this manner we have demonstrated that washed human platelets are able to synthesize the major phosphoglycerides including phosphatidylinositol, phosphatidyl serine, phosphatidyl choline, and phosphatidyl

[1] IUPAC-IUB. 1967. Nomenclature of lipids. *J. Biol. Chem.* **242**: 4845.

ethanolamine. In other experiments, we have shown that addition of thrombin to washed whole platelets has a striking effect, inhibiting glycerol incorporation into phosphatidyl choline and phosphatidyl ethanolamine, while stimulating incorporation of glycerol into phosphatidyl serine.

METHODS

Preparation of platelets. Blood from healthy volunteers was collected in 500 ml volumes, in plastic bags containing ACD,[2] and centrifuged for 6 min at 2300 rpm at 5°C in a Sorvall RC-3 centrifuge. All samples were processed immediately after the blood was drawn with subsequent steps being carried out using either plastic or siliconized equipment. The platelet-rich plasma was removed from the erythrocyte pellet and centrifuged for 25 min at room temperature at 100 *g* to further decrease erythrocyte and leukocyte contamination. The platelet button was resuspended and washed twice in buffer containing 0.154 M NaCl, 1.0 M $NaHCO_3$, 0.077 M Na_2 ethylenediaminetetraacetate (EDTA), pH 7.4, H_2O; 90 : 1.2 : 2 :6.8. The washed platelets were then suspended in the same buffer with 10^{-4} M glycerol-1,3-^{14}C (9 μc/μmole) at a platelet concentration of 1–2.5 × 10^9/ml. Incubations were carried out at 37°C in a Dubnoff shaker mixing at 70 rpm, and, after incubation, the platelet suspension was added to an equal volume of cold 10% trichloroacetic acid. The precipitate was then washed twice with 5% trichloroacetic acid, and extracted with 1 : 1 chloroform : ethanol according to the method of Hokin and Hokin (10). White cell contamination of platelet preparations was determined to be 1 white blood cell (WBC)/5 × 10^4 platelets, and in separate experiments in which leukocytes were incubated with glycerol-^{3}H, it was shown that such contamination could account for less than 1% of the total glycerol incorporation by platelets. Erythrocyte contamination was not a possible source of incorporation, as previous studies have shown that mature erythrocytes do not incorporate glycerol into lipid (11).

For subsequent studies on the thrombin effect, platelets were isolated as described above and washed twice in a buffer composed of 0.154 M NaCl, 0.154 M Tris-HCl, pH 7.4, 0.077 M Na_2EDTA, pH 7.4; 90 : 8 : 2, containing 1 mg/ml glucose. The platelets were then incubated with 10^{-4} M glycerol-^{14}C (9 μc/μmole), or 10^{-4} M glycerol-^{3}H (50 μc/μmole), in a buffer composed of 0.154 M NaCl, 0.154 M Tris-HCl, pH 7.4; 9 : 1, containing 1 mg/ml glucose. The lipids were precipitated and extracted as described above. Trypsin used in platelet incubations was Worthington 2 X crystallized trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) (12) to remove chymotryptic activity. Thrombin preparations used were Parke-Davis or Upjohn bovine thrombin, or thrombin of specific activity 5000 U/mg of tyrosine purified free of factor X^a activity by the method of Yin and Wessler (13). Human thrombin was purified by Dr. Kent Miller, New York State Public Health Service, Albany, N. Y. Thrombin preparations were assayed for their ability to clot fibrinogen just before use (14).

Chromatography. The total lipid extracts were chromatographed on Whatman SG81 silica gel bonded paper with diisobutyl ketone : acetic acid : 0.9% NaCl 40 : 25 : 5 (15). Phospholipids were identified after rhodamine 6G staining (15) by comparison with reference compounds. The spots were either eluted by the method of Abramson and Blecher (16) or by eluting in Brays solution in scintillation vials before counting. In these latter experiments, the recovery of radioactivity was about 50%. Some of the loss of radioactivity was due to two other radioactive spots which moved faster than phosphatidyl ethanolamine. These radioactive substances remain unidentified. The majority of the loss, however, is due to incomplete elution in Brays solution, since prior elution of the spots resulted in 80–90% recovery of radioactivity.

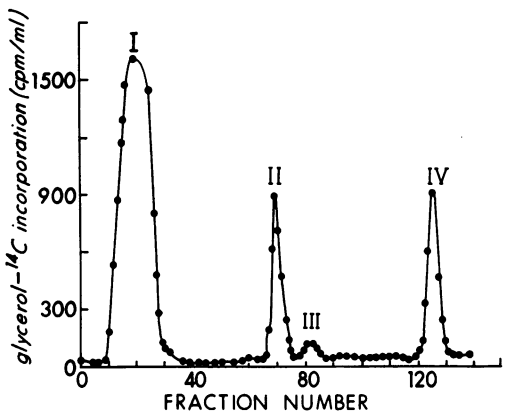

FIGURE 1 Dowex 1 acetate chromatography of glyceryl phosphoryl derivatives. See Methods for details. Peak I contains glycerylphosphorylcholine, glycerylphosphorylethanolamine, and glycerol; peak II, glyceroylphosphorylinositol; peak III, glycerylphosphorylserine; and peak IV, sn-glycerol 3-phosphate.

Alternatively, the lipids from 1 to 2 U of platelets were subjected to the alkaline alcoholysis procedure of Dawson (17). After alcoholysis, reaction mixtures were neutralized by addition of Dowex 50 hydrogen to pH 6.0. The glyceryl phosphoryl derivatives formed were then applied to a Dowex 1 acetate column (1.5 × 25 cm), and, after washing with one column volume of distilled water, the glyceryl phosphoryl derivatives were eluted using 500 ml of sodium acetate, pH 6.0, in a linear gradient (0–0.85 mole/liter) and 3 ml fractions were collected. Glycerylphosphorylcholine (GPC), glycerylphosphorylethanolamine (GPE), and free glycerol, which are not retained by the column, were further resolved by descending chromatography using Whatman 1 paper with phenol saturated with 0.1% w/v NH_3 (18), and by ascending chromatography with propanol : ammonia : H_2O, 60 : 30 : 10 (19). The radioactive GPC and GPE were then eluted from the paper with H_2O. The other glycerylphosphoryl derivatives, which eluted as separate peaks from the Dowex column (Fig. 1), were glycerylphosphorylinositol (GPI), glycerylphosphorylserine (GPS), and sn-glycerol-3-phosphate. The latter peak was identified by cochromatography with carrier sn-glycerol-3-phosphate on Dowex 1 acetate and by enzymatic assay of the fractions using glycerophosphate dehydrogenase (20). Peak III was identified as GPS by hydrolysis of this compound to free serine by the method of Dawson (18); the released serine was then converted to its dinitrophenyl derivative by the method of Rao and Sober (21), and the compound formed was identified as dinitrophenyl serine by its cochromatography with authentic dinitrophenyl serine on thin-layer plates in chloroform : *t*-amyl alcohol : acetic acid : 70 : 30 : 3 (22). Ninhydrin assays of the GPS indicated 0.72 μmole amino

[2] ACD, acid citrate dextrose, NIH formula A.

nitrogen/μmole phosphorus (23). The GPI peak was identified by demonstrating cochromatography of the radioactive GPI formed with a reference sample of GPI prepared from authentic phosphatidylinositol. All GP derivatives thus separated by the combination of column and paper chromatography were assayed for phosphorus by a micromodification of the method of Ames and Dubin (24).

Materials. Glycerol-1,3-^{14}C and glycerol-2-^{3}H were purchased from New England Nuclear Corp., Boston, Mass. Standard bovine phosphatidyl serine, phosphatidylinositol, phosphatidyl ethanolamine, and phosphatidyl choline were purchased from Applied Science Laboratories Inc., State College, Pa. Soybean trypsin inhibitor was purchased from Mann Research Laboratories Inc., New York.

RESULTS

Glycerol incorporation. In preliminary experiments, it was demonstrated that washed platelets incorporate glycerol-^{3}H into platelet lipids. The saturating concentration of glycerol was 10^{-4} moles/liter, and this concentration was used in all subsequent experiments. Incorporation of glycerol was linear with time for 2 hr, and over 75% of the glycerol-^{14}C taken up by platelets was incorporated into lipid.

Glycerophosphatide synthesis. The radioactive lipid formed from incubation of the platelets from 500 ml of blood with glycerol-^{14}C was subjected to alkaline alcoholysis, and the resulting water-soluble glycerylphosphoryl derivatives were separated as described above. The total incorporation in this experiment was 135,000 cpm in 2×10^{10} platelets. After alcoholysis, 6% of the counts were found in glycerol, indicating that this incorporation was into neutral lipids and the remainder into phospholipids. The incorporation of glycerol-^{14}C into the various glycerylphosphoryl derivatives from this experiment is shown in Table I. The over-all recovery of radioactivity in these derivatives after the alcoholysis and column procedure was 74%. To insure that the recovery for the individual compounds was similar, an aliquot of the total lipid extract was separated into the various phospholipids by chromatography on silica gel paper, and the results of this separation are also shown in Table I. The incorporation into phospholipid, as measured by the two methods, was quite similar, and the recoveries of the individual glycerylphosphoryl derivatives in the alcoholysis procedure closely reflected the over-all recovery of radioactivity, indicating that the incorporation and the specific activity of individual glycerylphosphoryl derivatives after alcoholysis accurately reflect the pattern of incorporation into phospholipid. The incorporation into specific phospholipids varied between batches of platelets; for example, in another experiment the specific activities obtained were: GPE, 3,500 cpm/μmole phosphorus, GPS, 1,200 cpm/μmole phosphorus, GPI, 25,000 cpm/μmole phosphorus, and GPC, 12,800 cpm/μmole phosphorus. When platelets were stored more than 8 hr before incubation, a greater proportion of label incorporation appeared in sn-glycerol 3-phosphate, suggesting that some of the enzymes which convert phosphatidic acid to the other glycerophosphatides are unstable. For this reason, incubations were routinely carried out immediately after platelet preparation, and most experiments were completed within 3–4 hr after blood was drawn. The amount of sn-glycerol 3-phosphate obtained after alcoholysis probably overestimates the amount of phosphatidic acid formed, since breakdown of the other phosphatides may lead to sn-glycerol 3-phosphate formation (17). This interpretation is supported by the fact that the radioactivity in sn-glycerol 3-phosphate in Table I was greater than that found in phosphatidic acid as measured in the unhydrolyzed compound.

The specific activity of phosphatidic acid could not be measured directly because the small amount of this compound present did not allow accurate phosphate determinations. However, it is clear that the specific activity of phosphatidic acid was higher than that of the other phosphatides.

TABLE I
Glycerol Incorporation into Phosphatides

	Alkaline alcoholysis*			Silica gel paper	
	μmoles P_i	cpm	cpm	cpm	
	2×10^{10} platelets	2×10^{10} platelets	μmole P_i	2×10^{10} platelets	Recovery in alcoholysis
					%
Phosphatidyl choline	2.55	56,200	22,000	67,500	83
Phosphatidyl ethanolamine	1.65	9,700	5,900	14,800	66
Phosphatidyl serine	1.46	2,550	1,750	3,000	85
Phosphatidylinositol	0.77	14,200	18,400	17,800	80
Phosphatidic acid	—	15,600	—	3,700	

* Measured as GP derivatives as described in Methods.
Platelets isolated from 1 U of fresh blood were incubated with glycerol-^{14}C for 1 hr as described in Methods.

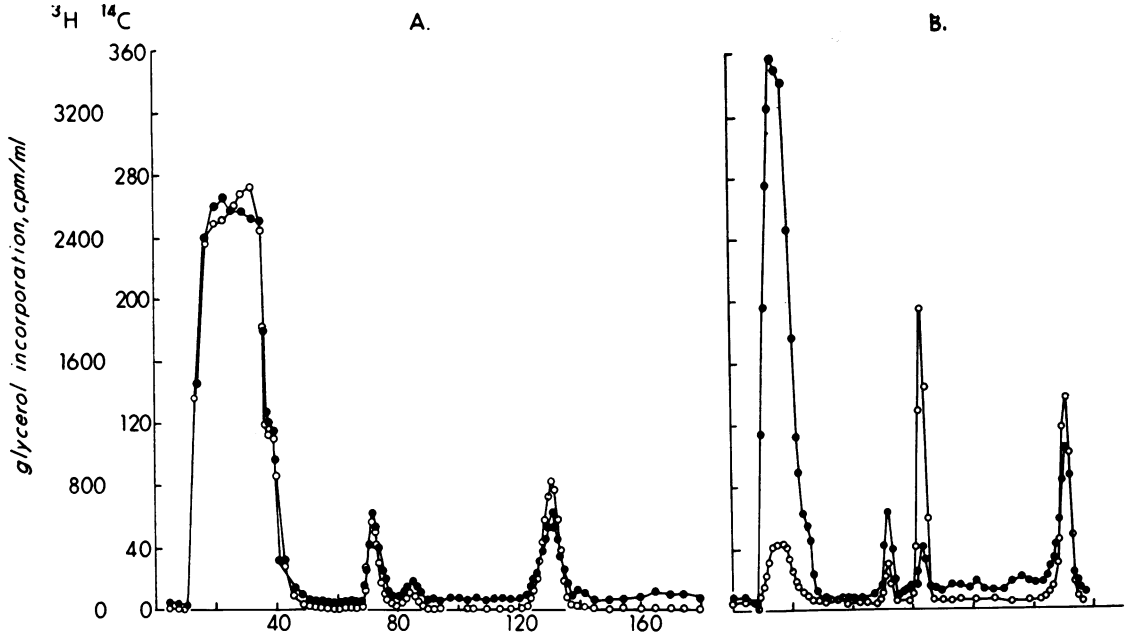

FIGURE 2 Dowex 1 acetate chromatography of glycerylphosphoryl derivatives. Conditions as described in Methods. A. Control experiment: ● ● ● glycerol-^{14}C, ○ ○ ○ glycerol-^{3}H. B. Thrombin experiment: ● ● ● glycerol-^{14}C, ○ ○ ○ glycerol-^{3}H plus Parke-Davis thrombin 1 U/ml and $CaCl_2$ 2.5 mmoles/liter.

Specific activities of the phospholipids formed could not be determined from the paper chromatography system since this one-dimensional system does not adequately separate sphingomyelin from the phosphatidyl choline (PC) and phosphatidylinositol (PI) spots, and, furthermore, the various lyso compounds are not resolved in this system. The radioactive spots corresponding to phosphatidylserine (PS), phosphatidyl ethanolamine (PE), PI, and PC were sufficiently well resolved in this system to allow accurate assessment of total incorporation into each of these compounds. The fact that 80% of the total counts incorporated into lipid could be recovered as glycerol or as glycerylphosphoryl derivatives after alcoholysis indicates that the label is being incorporated largely as glycerol and is not being significantly converted to fatty acid before incorporation.

Thrombin effect on glycerol incorporation into lipid. It was difficult to interpret the effect of thrombin on total glycerol incorporation into either washed platelets or platelets in platelet-rich plasma. In some experiments, a slight initial stimulation of incorporation was noted in the first 2–5 min as compared with incorporation by platelets incubated in the absence of thrombin. This early effect was variably present and was not further investigated. In all experiments, there was a subsequent decrease in incorporation so that by 30 min, the total incorporation had decreased to ⅓ that of control platelets. In all incubations comparing thrombin-treated with control platelets, it was necessary to add glucose to the incubations, since, in the absence of glucose, adenosine triphosphates (ATP) levels fell by 20% in 30 min, even without thrombin. This decline could be prevented by the addition of glucose (1 mg/ml).

Effect of thrombin on pattern of glycerophosphatide synthesis. The effect of thrombin on glycerol incorporation into specific glycerophosphatides was first examined

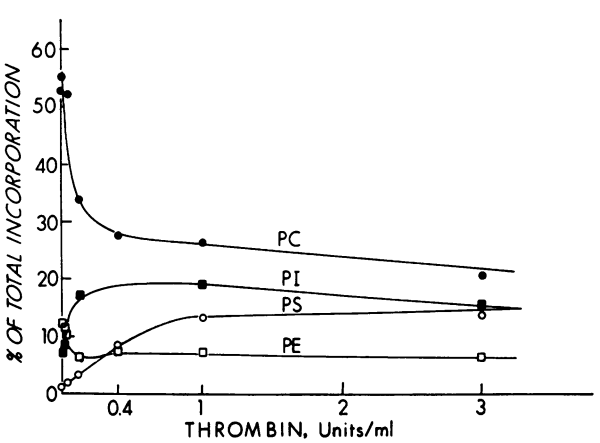

FIGURE 3 Pattern of glycerol-^{3}H incorporation as a function of thrombin concentration. $CaCl_2$, 2.5 mmoles/liter, was included in all reactions with 3×10^{9} platelets in 2 ml. Reaction mixtures were incubated 30 min as described in Methods.

in parallel double-label experiments shown in Fig. 2. Platelets from 2 U of blood were isolated and used in four equal aliquots. In the control experiment, one aliquot of platelets was incubated with glycerol-^{14}C and another with glycerol-^{3}H for 30 min. The ^{14}C- and ^{3}H-labeled platelets were then mixed and their lipids extracted, subjected to alcoholysis, and fractionated. In the thrombin experiment, one aliquot contained glycerol-^{3}H, thrombin, and calcium while the other contained only glycerol-^{14}C. After incubation, the ^{14}C- and ^{3}H-labeled batches were again mixed and treated as described above. As seen in the control experiment, Fig. 2 A, there is a close correspondence between ^{3}H and ^{14}C recoveries for each compound. The ^{3}H : ^{14}C ratio obtained from silica gel paper also indicated a constant recovery of each compound although the ratios were somewhat lower possibly due to some quenching of ^{3}H after staining with rhodamine G and elution from paper. The ratios ^{3}H : ^{14}C obtained from paper were: PS, 7.3; PI, 7.4; PE, 7.0; and PC, 7.5. In the second experiment, the glycerol-^{14}C platelets served as a control for losses during alcoholysis and isolation and could be compared to thrombin-treated platelets incubated with glycerol-^{3}H. Fig. 2 B indicates the change in the pattern of synthesis resulting from thrombin treatment. There was a decrease in incorporation into phosphatidyl choline and phosphatidyl ethanolamine while a striking increase in incorporation into phosphatidyl serine occurred. As stated above, it is not possible to evaluate the apparent rise in sn-glycerol 3-phosphate activity, as this may have arisen from degradation of phosphatidyl serine. In this

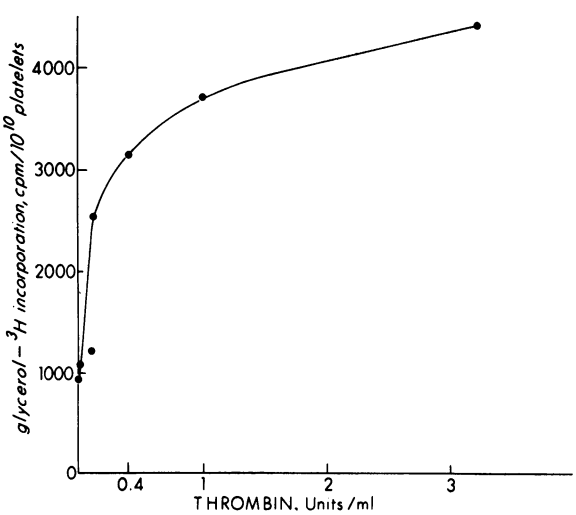

FIGURE 4 Effect of Parke-Davis bovine thrombin concentration on glycerol-^{3}H incorporation into PS. Phospholipids were isolated and separated on silica gel paper as described in Methods. Reaction mixtures contained 3×10^{9} platelets, thrombin, and 2.5 mM $CaCl_2$ in a volume of 2 ml and were incubated for 30 min.

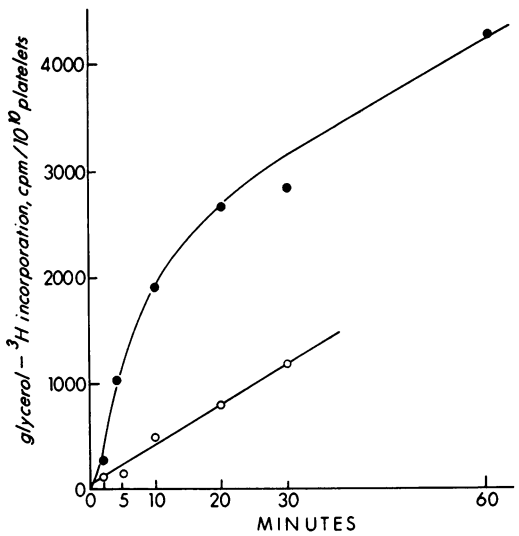

FIGURE 5 Time course of glycerol-^{3}H incorporation into PS in control ○ ○ ○ and thrombin (3 U/ml) treated platelets ● ● ● . Reaction mixtures contained 2.5 mM $CaCl_2$ and reactions were started by the addition of 4×10^{9} platelets in a final volume of 2 ml.

experiment, there was no apparent change in phosphatidylinositol; however, in some other experiments a 2- to 3-fold increase in ^{3}H : ^{14}C ratios was noted. The ^{3}H : ^{14}C ratios noted in this experiment were GPC : GPE, 1.3; GPI, 5; and GPS, 49. Thus, the relative stimulation of incorporation into phosphatidyl serine was 5-fold, based on the change in ^{3}H : ^{14}C ratio between experiments shown in Fig. 2 A and B, although the absolute increase was less than this due to a decrease in total incorporation of glycerol in the thrombin-treated aliquot. Similar results were seen when the labels were reversed, adding thrombin to the glycerol-^{14}C incubation. Incubation of platelets with calcium alone caused no effect, but calcium did potentiate the effect of thrombin.

The effect of changing thrombin concentration on the pattern of glycerophosphatide formation was next studied as shown in Fig. 3. This experiment demonstrates that the major effect of thrombin occurs when 1 U/ml of thrombin is incubated with platelets, even though the concentration of platelets is 5- to 10-fold greater than in whole blood. The pattern seen in this experiment again demonstrates a relative decrease in PC and PE formation with, in this case, a 2-fold relative increase in PI formation and a 10-fold relative increase in PS formation. The absolute increase in incorporation into PS is less than 10-fold because the total glycerol incorporation was decreased 3-fold in the presence of thrombin. Fig. 4 shows the absolute increase in PS formation with increasing thrombin concentration. In this experiment, there was a 4-fold absolute increase in glycerol incorporation into PS. It is difficult to determine whether it is

more appropriate to consider the thrombin effect on PS in relative or in absolute terms. Thus, the decreased total incorporation of glycerol-^{3}H into phospholipids after thrombin treatment may result from dilution of sn-glycerol 3-phosphate from endogenous unlabeled precursors (see Discussion). If this is true, it is more appropriate to consider the stimulation of PS formation in relative terms. The time course of the thrombin effect (Fig. 5) demonstrates that thrombin has a rapid effect on the rate of glycerol incorporation into PS, which is maximal by 2 min. This increased rate (7-fold greater than the control in this experiment) is transient, however, and by 20 min, the rate of incorporation of glycerol into PS has nearly returned to control levels. When platelets were preincubated with thrombin for 20 min, recovered from the incubation mixture by centrifugation, and resuspended in glycerol-^{3}H in the absence of thrombin, no burst of incorporation was seen. Rather, incorporation of glycerol into PS was parallel to that in control platelets, and incorporation into PC and PE remained at half the level of control platelets. Thus, it appears that thrombin causes a permanent decrease in the incorporation of glycerol into PC and PE but a transiently increased incorporation into PS.

Specificity of thrombin effect. Since the thrombin used for the experiments described above was relatively crude, we next studied the specificity of this effect using purified bovine thrombin obtained from Doctors S. Wessler and E. T. Yin (13). The results of these experiments are shown in Table II. In platelets incubated without thrombin, from 1 to 1.5% of the radioactivity incorporated was found in PS. When Upjohn thrombin (3 U/ml) was used, there was a 2-fold absolute increase in incorporation of glycerol-^{3}H into PS, and the relative incorporation was increased 8-fold. A similar increase was seen using purified thrombin at a level of 3 U/ml. This thrombin was effective at the same levels as the crude Upjohn thrombin, even though it had been purified 15-fold. In other experiments studying the effect of increasing concentrations of purified thrombin on glycerol incorporation into PS, the maximal effect was reached by about 1 U/ml. Therefore, if the effect noted is due to some substance other than thrombin in the preparations used, it must copurify with thrombin over 15-fold. This purified thrombin has been shown by Wessler and Yin to be free of other clotting factors, especially factor X^a, which is a major contaminant of the crude thrombin preparations. It is of interest that X^a purified from commercial thrombin, but free of thrombin activity, did not produce an increase in PS formation (experiment 6, Table II). In another experiment, the effect of an insoluble polyanionic derivative of thrombin (25) on glycerol incorporation into PS was tested. This preparation was no longer capable of clotting fibrinogen, although some esterase activity against model substrates such as tosyl-arginyl-methyl ester remained. The results obtained with this substance showed that there was a 2-fold stimulation of incorporation of glycerol into lipid but no relative stimulation of PS formation. The significance of this result remains to be elucidated. As further evidence that the effects noted are relatively specific, platelets were incubated in the presence of albumin and fibrinogen with no increase in PS formation, although there was some increase in the total incorporation of glycerol into lipid.

Effect of human thrombin on glycerol incorporation into phosphatidyl serine. The effect of human thrombin on glycerol incorporation into phospholipid was studied in order to insure that the effects noted above were not secondary to the species difference between the platelets and thrombin used. Highly purified human thrombin produced effects essentially identical to those seen with bovine thrombin with a maximal effect being produced by 1 U/ml as shown in Table III (experiments 9–11).

TABLE II

Specificity of Thrombin Effect on Phosphatidyl Serine Synthesis

Experiment No.	Addition	Total glycerol-^{3}H incorporation	Net phosphatidyl serine synthesis	
			cpm/10^{10} Platelets	% of total incorporation
		cpm/10^{10} platelets		
1 *a*	None	126,000	1360	1.1
1 *b*	None	113,000	1660	1.5
2	Upjohn bovine thrombin, 3 U/ml	33,000	3180	9.7
3	Purified bovine thrombin, 3 U/ml	32,000	3270	10.2
4	Fibrinogen, 2 mg/ml	222,000	1570	0.7
5	Bovine serum albumin, 0.17 mg/ml	150,000	1440	1.0
6	Bovine factor X^a, 12 U/ml	115,000	1640	1.4

Reactions were started by the addition of platelets and were incubated for 30 min at 37°C as described in Methods. The platelets used for this experiment were obtained from 2 U of blood and were incubated in 2 ml reaction mixtures of a concentration of 3×10^9/ml.

Higher levels of human thrombin produced no added stimulation of glycerol incorporation into PS, although there was further decrease in the total incorporation of glycerol into lipid. The fact that the effect on phospholipid synthesis was again seen at physiological levels of thrombin despite the fact that this thrombin was purified by a technique quite different from that used to purify the bovine thrombin is further support for the hypothesis that the effects produced are caused by thrombin itself.

Effect of trypsin on glycerol incorporation into phosphatidyl serine. Thrombin is a proteolytic enzyme which not only clots fibrinogen but also causes platelets to aggregrate and undergo "viscous metamorphosis." Trypsin has also been shown to aggregate washed platelets (26) in a manner similar to that seen with thrombin, and, thus, its effect on glycerol incorporation into PS was examined. As shown in Table III (experiments 1 through 7), trypsin also produces marked stimulation of glycerol incorporation into PS. In this experiment, there was a 5- to 6-fold increase over the control incubation in the relative incorporation into PS. The maximal effect produced by trypsin occurred at levels of about 0.01 mg/ml. In other experiments using trypsin, the relative incorporation into PS was stimulated up to 10-fold. When trypsin was preincubated with soybean trypsin inhibitor (Table III, experiments 6–8), the effect of trypsin was abolished, suggesting that the proteolytic action of trypsin may be required to produce the effect on PS formation. Furthermore, soybean trypsin inhibitor alone had no effect on PS formation.

Estimating the amount of thrombin necessary to produce the maximal effect on phospholipid synthesis from the specific activity of the most highly purified thrombin preparations (60,000–70,000 U/mg tyrosine) (14) suggests that thrombin is about 50-fold more effective than trypsin in stimulating PS synthesis on a molar basis.

It is difficult to be certain that the increased incorporation of glycerol into phosphatidyl serine actually reflects an increase in the rate of net synthesis of PS when studying intact cells. It is conceivable that the effect observed might be due to the sudden expansion of a rapidly turning over pool of PS within the cell resulting from decreased utilization of PS. Since the specific activity of PS (Table I) is very low, it is clear that the majority of platelet PS is not metabolically active. Thus, one must postulate a compartmentalized smaller metabolically active pool to explain our results on this basis. To examine this possibility, we performed a "pulse-chase" type experiment in which platelets were incubated with glycerol-^{3}H for 30 min and the radioactive glycerol was then diluted 30-fold by the addition of an excess of unlabeled glycerol and incubation continued for an additional 30 min. In this experiment, the radioactivity in PS remained constant after the first 30 min and did not decrease significantly

TABLE III

Effect of Trypsin and Human Thrombin on Phosphatidyl Serine Synthesis

Experiment No.	Addition	Total glycerol-^{3}H incorporation	Net phosphatidyl serine synthesis	
			cpm/10^{10} platelets	% of total incorporation
		*cpm/10^{10} platelets**		
1	None	170,000	1408	0.8
2	Trypsin, 0.002 mg/ml	148,000	3450	2.3
3	0.01 mg/ml	99,000	4960	5.0
4	0.05 mg/ml	98,000	5900	6.0
5	0.2 mg/ml	89,000	4680	5.2
6	Soybean trypsin inhibitor, 0.08 mg/ml	140,000	1400	1.0
7‡	Trypsin 0.05 mg/ml and soybean trypsin inhibitor 0.08 mg/ml	154,000	1708	1.1
8	Human thrombin 0.1 U/ml	135,000	1600	1.2
9	0.4 U/ml	129,000	3250	2.5
10	1.0 U/ml	105,000	5780	5.5
11	Bovine thrombin 3 U/ml	50,700	3131	6.2

Reactions were started by the addition of platelets and were incubated for 30 min at 37°C as described in Methods. Human thrombin was obtained from Dr. Kent Miller, New York Public Health Service, Albany, N. Y.

* The platelets used in this experiment were obtained from 2 U of blood and were incubated at a concentration of 3×10^9/ml in a total volume of 2 ml/reaction mixture.

‡ In experiment 7, trypsin and soybean trypsin inhibitor were preincubated together 15 min before platelet addition.

during the "chase," suggesting that the PS pool does not turn over rapidly. This experiment does not unequivocally prove that the increased incorporation into PS represents increased synthesis, however, since the nature of the potential interconversion of the various phosphatides has not been elucidated in platelets, making a simple interpretation of this experiment difficult. This problem can potentially be approached directly by studying the effect of thrombin on individual enzymes involved in platelet phosphoglyceride synthesis. In preliminary experiments using platelet extracts, thrombin produced no effect on the reaction which converts sn-glycerol 3-phosphate and palmityl CoA to phosphatidic acid.

DISCUSSION

It is clear from previous studies that platelets have an active lipid metabolism. They are capable both of *de novo* fatty acid synthesis and of chain elongation of preformed fatty acids (3). Previous studies using ^{32}P incorporation suggested that circulating platelets might synthesize phospholipid as well (6). In these studies, Firkin and Williams suggested that phosphatidic acid and phosphatidyl inositol were metabolically the most rapidly turning over lipids in platelets, although they were unable to determine whether net synthesis occurred. The present study clearly demonstrates that platelets are able to synthesize the major classes of phospholipid *de novo*. The results of glycerol incorporation indicate that phosphatidic acid, PC, and PI are metabolically active in platelets relative to PE, and that PS formation occurs to a much more limited extent. While glycerol incorporation indicates that net synthesis occurs, the relative magnitude of this pathway cannot be determined from our studies. The amount of total phospholipid synthesized from glycerol in these experiments is extremely small compared with the total lipid content of platelets, but this does not necessarily indicate that the pathway is unimportant in platelet function. The incorporation noted is certainly a minimal figure as glycerol transport or glycerokinase may be rate-limiting in these studies while the more natural pathway for lipid synthesis from sn-glycerol 3-phosphate formed from dihydroxyacetone phosphate might be potentially more active. Furthermore, the degree of dilution of labeled sn-glycerol 3-phosphate by endogenously formed unlabeled sn-glycerol 3-phosphate is unknown.

Preliminary reports of study of the individual enzymes of phospholipid synthesis in platelets have described the properties of the enzymes which convert sn-glycerol 3-phosphate to phosphatidic acid (3) and those which subsequently convert phosphatidic acid to PI (7). Further studies are required to define the reactions whereby the other phosphatides are formed.

The mechanism of the thrombin effect and its physiological significance remain unexplained. While the inhibition of glycerol incorporation into PC and PE produced by thrombin might be ascribed to "nonspecific" effects of proteolysis on the enzymes of lipid synthesis or by the reduced availability of glycerol to the mass of aggregated platelets, it is difficult to explain the striking stimulation of PS formation on this basis. It is also possible that thrombin does not decrease the net synthesis of phosphatides as would appear from the decreased incorporation of glycerol-^{3}H into total platelet lipid. Thrombin causes a marked increase in glycogenolysis and glucose oxidation via glycolysis in platelets (27–29). Thus, it is possible that the reduced incorporation of glycerol-^{14}C into phospholipid is produced by dilution of the sn-glycerol 3-phosphate pool by the increased flow of unlabeled substrates through the glycolytic pathway. This would imply that the true stimulation of PS synthesis after thrombin treatment might be 10- to 20-fold as reflected by the relative increases in PS formation. Another possible explanation is that thrombin induces some conformational change on the platelet membrane surface possibly by proteolytic action. This change might alter the spatial configuration of the enzymes of phospholipid synthesis leading to altered patterns of synthesis. Other experiments have shown that thrombin has numerous other actions on platelet metabolism. Thus, thrombin causes an increase in ATPase activity, increased lactate production (27), and a marked fall in intracellular adenosine diphosphate (ADP) and ATP levels (27, 29–31). It is of interest in this regard that the time course of the thrombin effect on changes in intracellular nucleotides parallels the effect on phospholipid synthesis with rapid changes occurring almost immediately and being complete within 20 min (29). All of these effects may be mediated indirectly by some single effect of thrombin on the platelet membrane. Alternatively, thrombin may act directly on one or more of the enzymes of phospholipid synthesis. The increased rate of PS synthesis could be involved in the repair of platelet membrane damage produced by thrombin, although the small amount of PS synthesized would seem unlikely to be sufficient to provide any net structural increase in membrane. It is also possible that the burst of PS synthesis is involved in the activation or formation of some factor required for coagulation, although further study is required to evaluate this point. In this regard, Marcus has shown that PS seems to be the lipid which can best replace platelet membranes (32), even though no isolated lipid is as effective as platelet membranes themselves (33).

The exact mechanism by which thrombin produces its effect on platelet lipid synthesis also remains unexplained. The fact that the effect on PS synthesis can be

produced by trypsin suggests that proteolysis may be involved in this reaction. This is further supported by the fact that soybean trypsin inhibitor markedly reduces the trypsin effect, and an insoluble thrombin derivative which does not clot fibrinogen has also lost the ability to stimulate PS formation.

It is conceivable that the effect noted here is produced by some substance in the thrombin preparations used other than thrombin itself, and further experiments are required to settle this point. In recent experiments reported by Ganguly (34) using human thrombin, it was shown that platelets contain more than one protein which is subject to proteolysis by thrombin. These other proteins were clearly not fibrinogen, and whether this proteolytic effect is due to thrombin itself or to other similar proteolytic enzymes has not been resolved. In addition, the functional nature of these thrombin-sensitive proteins remains unknown.

In a preliminary report, Nishizawa and Holder (35) reported that platelet aggregation produced by either ADP or collagen caused an increase in ^{32}P incorporation into PS. We have carried out preliminary experiments studying the effects of ADP on washed platelets. These experiments are difficult to interpret since at times washed platelets do not aggregate in the presence of ADP. In any case, when ADP was added to platelets incubated with glycerol-^{3}H in a double-label experiment, no effect on the pattern of lipid synthesis was noted and no relative increase in PS formation occurred.

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