Production of Diglyceride from Phosphatidylinositol in Activated Human Platelets

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ABSTRACT Human platelets generate diglyceride within 5 s of exposure to thrombin. Production of diglyceride is transient. 15 s after the addition of thrombin, the levels of diglyceride have increased up to 30-fold, but decrease thereafter. Prior incubation of platelets with 2 mM dibutyryl cyclic AMP prevents both the generation of diglyceride and the secretion of serotonin. Acetylsalicylic acid (100 μg/ml), which completely inhibits prostaglandin endoperoxide synthesis, does not block diglyceride production and serotonin secretion induced by thrombin.

Based on studies examining the incorporation of [^{3}H]arachidonic acid into diglyceride of prelabeled platelets exposed to thrombin, it is concluded that neither phosphatidic acid nor triglyceride is the source of the diglyceride. Phosphatidylinositol appears to be the most likely source, both because its loss of radiolabel is sizable and rapid enough to account for the appearance of radiolabel in diglyceride, and because a phosphatidylinositol-specific phosphodiesterase, described in this report, exists in platelets. The phosphatidylinositol-phosphodiesterase, which produces diglyceride and inositol phosphate, requires Ca^{+2} and shows optimal activity at pH 7. The enzyme does not act upon phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine.

INTRODUCTION

The human platelet shares with other secretory cells a "phosphoinositide effect" (1, 2), broadly defined as an alteration in the metabolism of phosphatidylinositol (PI)[1] preceding and paralleling secretion. In tissues such as pancreas, cerebral cortex, and parotid gland, stimulation by acetylcholine elicits a breakdown of PI (3–5). The secretory response of platelets is essentially complete within 30 s (6). Therefore, those changes in lipid metabolism that may participate in promoting secretion must be well underway within this period. The rapid generation of cyclic endoperoxides derived from arachidonic acid released from PI and phosphatidylcholine (PC) is one such change (7–10). Additional short-term effects characterized thus far in platelets exposed to agents such as collagen, ADP, and thrombin have been an increased incorporation of [^{32}P]orthophosphate into phosphatidic acid and polyphosphoinositides (11) and a decrease in the amount of previously radiolabeled PI (12).

This report describes a rapid, transient generation of diglyceride in human platelets exposed to thrombin. Evidence is presented as well for the existence of a PI-specific phosphodiesterase in platelets which requires calcium ions for full activity and which may be involved in the turnover of PI associated with the secretory process.

Inhibitors of platelet cyclo-oxygenase, such as acetylsalicylic acid, have minimal effect on the release reaction induced by thrombin, however, N^6-$O^{2'}$-dibutyryl cyclic AMP (dBcAMP) prevents such release. The production of diglyceride by platelets was examined in the presence of acetylsalicylic acid and in the presence of dBcAMP in order to dissociate, if possible, the production of diglyceride from that of cyclic endoperoxides. The postthrombin formation of diglyceride appears to correlate with the occurrence of secretion, rather than with cyclic endoperoxide production.

METHODS

Preparation of cells and resolution of lipids. Platelet concentrates were provided by the Red Cross, within 24 h of phlebotomy, from normal donors. In some cases, blood was drawn in the laboratory from normal human volunteers and anticoagulated with acid-citrate-dextrose (13). After a preliminary centrifugation at 150 *g* for 1 min (platelet concen-

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[1] *Abbreviations used in this paper*: ASA, acetylsalicylic acid; dBcAMP, N^6-$O^{2'}$-dibutyryl cyclic AMP; DG, diglyceride; DOC, deoxycholate; HHT, 12-L-hydroxy-5,8,10 heptadecatrionoic acid; 5-HT, 5-hydroxytryptamine (serotonin); PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PRP, platelet-rich plasma; PS, phosphatidylserine; TG, triglyceride.

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trates) or 150 *g* for 15 min (whole blood), platelet-rich plasma (PRP) was mixed with EGTA to a final concentration of 1 mM. For studies on the metabolism of radiolabeled arachidonic acid or glycerol, PRP was incubated with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (12.5 μCi/30 ml PRP, 72 Ci/mmol) which had been bound to albumin as described previously (14), or with[(*N*)-2-^{3}H]glycerol (1 mCi/30 ml PRP, 6.48 Ci/mmol) for 20 min at 37°C. Otherwise, such an incubation was omitted. Platelet suspensions were then washed three times with a Tris-citrate-bicarbonate buffer (15) containing 2 mM EGTA at pH 6.5 after centrifugation at 2,500 *g* for 5 min at 4°C. Care was taken to separate platelets from any contaminating erythrocytes and neutrophils at the bottom of the pellet by sacrificing part of the platelet pellet. Platelets were finally suspended in buffer containing 1 mM EGTA, pH 6.8, to a final concentration of 10^{10} platelets/ml. Cell concentrations were determined with the aid of a Coulter device (Coulter Electronics, Inc., Hialeah, Fla.). Suspensions contained <0.01% of leukocytes and erythrocytes. For studies examining secretion by platelets, unlabeled platelet suspensions were then incubated with [^{14}C]5-hydroxytryptamine (creatinine sulfate salt, 1 μCi/5 ml suspension) for 15 min at 37°C. On the average, 98% of this material was taken up by the platelets in this period, making further washing unnecessary.

Before analysis, platelet lipids were extracted by the Folch procedure (16). The lower phase of the Folch extract was separated by silica paper chromatography or on silicic acid (Unisil 100–200 mesh, Unisil Corp., Greenwich, Conn.). Neutral lipids from silicic acid columns were concentrated under vacuum and applied to 0.5-mm silica G plates (A. G. Merck Darmstadt, West Germany) containing 0.4 M $(NH_4)_2SO_4$. Plates were placed in equilibrated tanks containing dichloroethane:MeOH (196:4 vol/vol), "system I," or benzene:diethyl ether:EtOH:NH_3 (100:80:4.0:0.2, vol/vol), "system II." Resolved lipids were made visible with I_2 and scraped off the plates for assay or were charred on a heated sand bath. Charred spots were quantitated by densitometry (Photovolt Corp., New York) in comparison with calibrating amounts of commercial triglyceride or diglyceride derived from human platelets as described below.

Diglyceride was generated from labeled platelet phospholipids for use in assessing recovery after the above extraction and chromatographic procedures, and as a standard for quantitative determinations on thin-layer plates. Methanol eluates from silicic acid columns (above), containing [^{3}H]-arachidonic acid or [^{3}H]glycerol-labeled phospholipids, were concentrated and suspended by sonication in 800 μl 50 mM Tris HCl buffer, pH 7.3, containing 0.4% delipidated bovine serum albumin. Sonicate (20 mg) was mixed with 100 μl 50 mM $CaCl_2$ and 25 μl phospholipase C from *Bacillus cereus* (50 μg/μl, Sigma Chemical Co., St. Louis, Mo.) and incubated for 60 min at 37°C. Lipids were extracted by the Folch procedure, and the $CHCl_3$ phase was resolved into neutral and polar lipids on silicic acid columns. [^{3}H]Diglyceride (DG) in the neutral lipid was identified on silica plates in both solvent systems I and II, in comparison with known standards of diolein, monoolein, tripalmitin, and arachidonic acid. The DG spots of such plates were scraped off and digested at 65°C for 2 h in closed tubes containing 5% KOH MeOH. After the mixtures were neutralized with HCl, lipids were extracted with $CHCl_3$ and rechromatographed on plates in solvent system II. The specific activity of the pure ^{3}H-labeled DG was determined by the use of a Cahn model 4400 electrobalance and scintillation spectrophotometry (Cahn Instruments, Division of Ventron Corp., Cerritos, Calif.).

Metabolic studies with intact cells. Washed platelets (10^{10} cells/ml) or erythrocyte-neutrophil suspensions (10^6 cells/ml) from preliminary centrifugation of PRP were incubated in a final volume of 1.05 ml with and without human thrombin (5–50 U/ml) for periods of up to 120 s. Siliconized glassware was used throughout. NaF (10 mM) was included in some mixtures. As a control for cellular damage, lactate dehydrogenase was assayed as previously described (17, 18). Incubations were terminated by the addition of 21 ml ice-cold $CHCl_3$:MeOH (2:1), and neutral lipids were resolved and quantitated as described above. Arachidonic acid oxidation products in the aqueous-MeOH phase were resolved by high pressure liquid chromatography.[2] Washed platelets whose lipids contained [^{3}H]arachidonic acid were incubated with or without dBcAMP (2 mM) for 15 min at 37°C or with or without acetylsalicylic acid (ASA; 100 μg/ml) for 5 min. Platelets were then incubated as above with 0.17 U thrombin/10^8 cells. Phospholipids were resolved by two-dimensional silica paper chromatography (8, 19). Radioactivity contained in the lipid spots was counted by scintillation spectrophotometry in H_2O-Aquasol (New England Nuclear) (8), and lipid phosphorus was assayed after digestion of parallel samples (19). Neutral lipids were resolved by thin-layer chromatography in solvent systems I and II. DG spots were charred, counted, or digested by KOH in MeOH as described, and rechromatographed. The altered distribution of radiolabeled material in I_2-visualized spots was determined.

Platelets whose lipids were labeled with [^{3}H]glycerol were also incubated as described for [^{3}H]arachidonic acid-labeled cells. The effect of thrombin on the release of [^{14}C]5-hydroxytryptamine from labeled platelets was also determined in the presence and absence of dBcAMP or ASA. Incubations were stopped with 2 vol of ice-cold buffer (15), pH 6.5, containing imipramine (6.7 μM) and EGTA (5 mM). Cells were sedimented at 2,500 *g* for 5 min at 4°C, and the release of ^{14}C-labeled material to the medium was assessed by scintillation counting.

Phospholipid phosphodiesterase assays. Washed platelets described above were suspended to a concentration of 3×10^9 cells/ml in buffer (15) containing 5 mM EGTA, pH 6.5. Platelet suspensions were sonicated in an ice-water bath with a microprobe for periods of 15 s. The total sonication time was 2 min. Similarly, suspensions of erythrocytes-neutrophils (3×10^5 cells/ml) were sonicated. Sonicates were spun at 100,000 *g* for 60 min at 4°C, and the supernatant solution was used in subsequent incubations.

Substrates for assay of phospholipid phosphodiesterase consisted of [^{3}H]myoinositol PI, [^{14}C]choline PC, phosphatidylethanolamine (PE), or phosphatidylserine (PS). [^{3}H]-Myoinositol PI was prepared according to a method described by Paulus and Kennedy (20), with chicken liver microsome suspensions. [^{14}C]Choline PC, PE, and PS were purchased from commercial sources. Microsomal suspensions were incubated with 1 mM $MnCl_2$, 10 μCi [(*N*)-2-^{3}H]-myoinositol (12.5 Ci/mmol), and 10 mM Hepes-NaOH, pH 7.4, at 37°C for 60 min. Lipids were extracted, washed of free [^{3}H]myoinositol and resolved on silicic acid columns into neutral lipid and labeled phospholipid fractions. The phospholipid was chromatographed on silica paper (8, 19). The radiolabel and phosphorus contents of PI were determined as were the amounts of other phospholipids present in the eluate (19). The remaining radiolabeled eluate was dried under vacuum and suspended with sonication in 25 mM Hepes-NaOH-50 mM NaCl,

[2] Russell, Francis A., and Daniel Deykin. Manuscript in preparation.

pH 7.0, to a final concentration of 0.42 mM [^{3}H]myoinositol PI. Also present in the phosphatide sonicates were 0.4 mM PS, 3.4 mM PC, and 2.1 mM PE.

Radiolabeled PC substrate was prepared by mixing unlabeled microsomal lipid extracts with commercial 10 μCi [*methyl*-^{14}C]choline PC, and suspending in buffer as described, yielding a final concentration of 0.42 mM [^{14}C]choline PC. Unlabeled PE and PS were each sonicated to Hepes buffer to a final concentration of 0.42 mM.

Assays for phosphatide phosphodiesterase activity contained Na deoxycholate (2.1 mg/ml) or Triton X-100 (1 mg/ml; Rohm and Hass Co., Philadelphia, Pa.); $CaCl_2$ (0–10 mM), 50 mM KCl, 21 nmol [^{3}H]myoinositol PI, [^{14}C]choline PC, PE, or PS, and up to 0.2 mg supernatant protein (fresh or heated at 100°C for 5 min) in a final volume of 200 μl. Some mixtures contained 10 mM NaF. Incubations continued for up to 60 min at 37°C, and were terminated by the addition of 1.0 ml $CHCl_3$:MeOH:HCl (100:100:0.6) and 0.3 ml 1 N HCl/5 mM EGTA. Part of the upper aqueous phase was assayed for radioactivity by scintillation spectrophotometry in H_2O-Aquasol (8). Other portions of the upper phase were applied to silica papers and chromatographed in two directions as before (8). The distribution of radiolabel among lyso-PI, inositol, and any PI present was determined for incubations that had included [^{3}H]myoinositol PI. Samples of the [^{3}H]myoinositol aqueous phase were also applied to Whatman no. 1 paper (Whatman, Inc., Clifton, N. J.) and resolved by descending chromatography in EtOH: 13.5 M NH_3 (3:2) as described by Dawson and Clarke (21), in comparison with the migration of inositol phosphate, [^{3}H]-myoinositol PI, [^{3}H]myoinositol, and glycerol phosphoinositol. Chromatograms were negatively stained for phosphate with 0.1% ethanolic $FeCl_3$:1% NH_4SCN in acetone (22) and counted by scintillation spectrophotometry.

The lower $CHCl_3$ phase (for PI, PS, and PE incubations) was concentrated and applied to a 0.5-g column of silicic acid washed with $CHCl_3$. Neutral lipid was resolved on 0.5-mm plates of silica G as described. Lipids were run in solvent systems I or II and were made visible by charring. DG was quantitated by densitometry in comparison with known standards. Other plates containing DG made visible by I_2 were scraped, and the lipid contained digested in KOH and rechromatographed as described. Phospholipid ([^{3}H]PI samples) was resolved by two-dimensional chromatography. The origin and regions corresponding to known lyso-PI and PI standards were assayed for ^{3}H.

Radioisotopes were obtained from New England Nuclear (Boston, Mass.) or Amersham Corp. (Arlington Heights, Ill.). Highly purified human α-thrombin (1 nM = 0.11 U/ml) was kindly provided by Dr. J. W. Fenton, II (New York State Department of Health). Lipid standards were supplied by Applied Science Labs (State College, Pa.), Supelco, Inc. (Bellefonte, Pa.), NuCheck Corp. (Elysian, Minn.), and Dr. John Pike of Upjohn Co. (Kalamazoo, Mich.). All solvents employed were spectrograde or redistilled. dBcAMP was purchased from Sigma Chemical Co.

RESULTS

Metabolic studies on intact cells

Time-dependent changes in human platelet phospholipids induced by thrombin. The production of DG by platelets exposed to 0.17 U thrombin/10^8 platelets was rapid. As illustrated in Fig. 1, within 5 s, 69% of the maximum amount of DG produced by the cell had been generated. The triglyceride content of

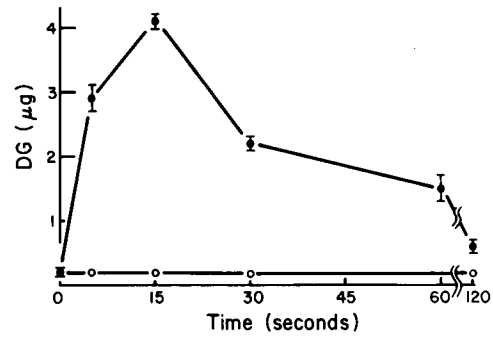

FIGURE 1 Generation of DG in response to thrombin. Washed human platelets (10^{10}/ml) were incubated with thrombin (0.17 U/10^8 cells) at 37°C. The incubations were stopped with 20 vol $CHCl_3$:MeOH (2:1). DG was quantitated by densitometry of charred spots on thin layer plates in comparison with known amounts of standards. Results are presented as averages for an experiment performed in duplicate. ●, +thrombin; ○, −thrombin.

platelets (not shown) was unaffected by thrombin. No response was observed for erythrocyte-neutrophil suspensions similarly incubated.

The time-course for the production of DG was duplicated by the course of labeling of this material by [^{3}H]arachidonic acid in platelets which had taken up the isotope before incubation with thrombin (Fig. 2A). Thrombin-free controls exhibited no change over the period examined. [^{3}H]Arachidonic acid-labeled triglyceride changed only slightly over this period, with or without thrombin. In the same experiment, labeled PI decreased, whereas 12-L-hydroxy-5,8,10 heptadecatrionoic acid (HHT; an oxidation product of arachidonic acid) and PA increased slightly in 5 s and reached a constant level after 30 s (Fig. 2B). Not shown is the finding that after 15 s the content of [^{3}H]-arachidonic acid had not changed significantly in PC,

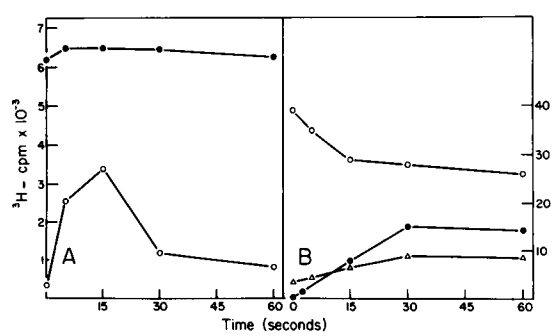

FIGURE 2 Change in content of [^{3}H]arachidonic acid in lipids with time, after exposure of labeled platelets to thrombin. Platelets (10^{10}/ml), preincubated with [^{3}H]arachidonic acid, were incubated with thrombin (0.17 U/10^8 platelets). Thrombin-free control results are not presented, but displayed no significant changes. (A) Changes in DG (○) and TG (●) labeling; (B) changes in PI (○), PA (△), and HHT (●) labeling.

PS, or diacyl-PE. After 30 s of exposure to platelets to thrombin, significant loss of [^{3}H]arachidonic acid from PC was detectable.

The DG produced in activated platelets was identified in comparison with the migration of commercial phospholipase C-generated standard DG after thin-layer chromatography. Triglyceride (TG), 1,2-DG, and arachidonic acid migrated in solvent system I with R_fs of 0.86, 0.31, and 0.25, respectively. The R_fs of TG, 1,2-DG, arachidonic acid, and HHT in solvent system II were 0.93, 0.71, 0.10, and 0.04. The DG produced by stimulated platelets was labeled either with [^{3}H]glycerol or with [^{3}H]arachidonic acid when prelabeled platelets were employed. Such DG was completely digested by mild akaline treatment, as was the DG standard. FFA was produced, containing [^{3}H]arachidonic acid.

The changes in specific activity ([^{3}H]arachidonic acid) for DG, TG, and PI are correlated in Table I. Values are calculated for the time of peak generation of DG, 15 s. If, based on a weighted average for the fatty acid composition of PI in human platelets (23), a molecular weight of 640 is assigned to the platelet DG, a conversion of mass to nanomoles can be made. A molecular weight of 840 is used for platelet TG, based on the fatty acid composition of human platelet TG (23). Mass values for PI were arrived at after phosphorus analysis. The calculations on a mole basis show that the loss of PI (15 nmol) can easily account for the gain in DG (6.1 nmol) observed. Further, labeled PI could account for the change in specific activity of DG. The specific activity of TG was low and did not change with time. Specific activity values for phosphatidic acid (PA) were not derived as it was not possible to measure PA phosphorus on silica papers, although radioactivity was consistently measurable. Such radioactivity migrated with known PA standards, and appeared in the neutral lipid region when the lipid on the spots was incubated under mild alkaline conditions and rechromatographed.

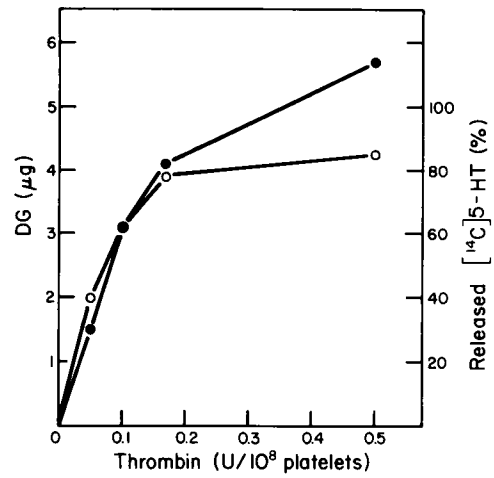

FIGURE 3 Generation of DG and release of [^{14}C]5-HT by human platelets in response to thrombin. In experiments monitoring DG, washed platelets (10^{10}/ml) were exposed to thrombin for 15 s at 37°C. Lipids were extracted, resolved, and quantitated as described for Fig. 1. Other suspensions of platelets, containing [^{14}C]5-HT were exposed to thrombin and sedimented. Release of [^{14}C]5-HT to the medium was determined. ●, DG; ○, percent release [^{14}C]5-HT.

A comparison of DG production and [^{14}C]5-hydroxytryptamine (HT) secretion in human platelets responding to various concentrations of thrombin is presented in Fig. 3. Near-maximum responses for both were attained with 0.17 U thrombin/10^8 platelets.

Contrasting effects of dBcAMP and ASA. Table II shows the effect of prior incubation of platelet suspensions with dBcAMP (2 mM) or ASA (100 μg/ml) on serotonin release and DG production as well as on the generation of [^{3}H]arachidonic acid-labeled products arising ultimately from cyclo-oxygenase activity. It is seen that both dBcAMP and ASA effectively eliminated the formation of such arachidonic acid products, ordinarily generated in response to thrombin. However, only dBcAMP inhibited serotonin secretion and DG production as well. It was also noted

TABLE I
*Specific Activity of [^{3}H]Arachidonic Acid-Labeled Lipid in Intact Platelets Exposed to Thrombin**

	t = 0	t = 15 s
	cpm/nmol	
DG	1,157	530
TG	49	51
PI	460	414

* Platelets (10^{10} cells/ml) containing [^{3}H]arachidonic acid-labeled lipids (no free arachidonic acid was present) were incubated with thrombin (0.17 U/10^8 platelets) for 15 s. Data represent the average±1.0% of an experiment performed in duplicate.

TABLE II
*Inhibitory Effects of dBcAMP and ASA on Changes Induced by Thrombin**

	Inhibition		
Inhibitor	DG	5-HT	HHT
		%	
dBcAMP	95±2	97±3	96±2
ASA	0±5	0±3	90±3

* The inhibition by dBcAMP or ASA in comparison with controls was derived from the expression 100 − (effect + inhibitor/effect − inhibitor)100. Results are the means±SE for two experiments performed in duplicate.

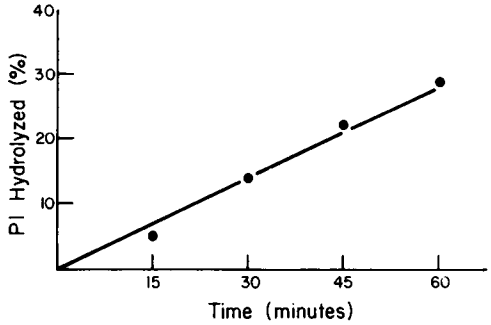

FIGURE 4 Hydrolysis of [^{3}H]myoinositol PI as a function of time. [^{3}H]Myoinositol PI was incubated with platelet sonicate supernate (0.5 mg/ml), pH 7.0, in the presence of 5 mM Ca^{+2} and 2 mg/ml DOC at 37°C. Incubations were concluded by the addition of $CHCl_3$-MeOH-HCl. The formation of water-soluble [^{3}H]myoinositol was determined after scintillation counting.

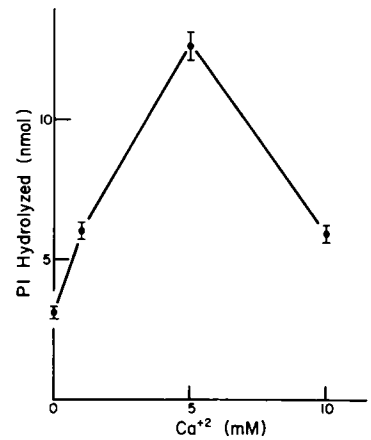

FIGURE 5 Hydrolysis of [^{3}H]myoinositol PI as a function of Ca^{+2} concentration. [^{3}H]Myoinositol PI was incubated with platelet sonicate supernate (1 mg/ml), pH 7.0, in the presence of 2 mg/ml DOC and varied amounts of Ca^{+2}. The formation of water-soluble [^{3}H]myoinositol was quantitated after incubation for 60 min at 37°C, as described for Fig. 4. Results are the means±SE of three experiments.

(not shown) that 10 mM NaF, which inhibits phosphatidic acid phosphatase in human platelets and other cells (24, 25) did not inhibit the formation of DG. Release of lactate dehydrogenase (2.1%) did not differ significantly from control levels.

Studies on disrupted cells

Phospholipid phosphodiesterase activity. Disrupted cell preparations were used to characterize the nature of the DG-generating enzyme system in platelets. Platelet sonicate supernates, incubated with [^{3}H]myoinositol PI, produced a lipid that migrated with R_fs corresponding to those of 1,2-DG in solvent systems I and II, and which yielded FFA upon mild alkaline hydrolysis. No such material was found in incubated supernate or in [^{3}H]myoinositol PI solutions incubated in the absence of supernate. The hydrolysis of [^{3}H]myoinositol PI by the supernate from platelet sonicates to water-soluble labeled material and DG was dependent upon the presence of both deoxycholate (DOC) and Ca^{+2}. The presence of 10 mM NaF affected neither the production of DG nor that of water-soluble material. In the absence of both DOC and Ca^{+2}, no hydrolytic activity was detectable Omission of either DOC or Ca^{+2} from the incubation resulted in little activity. Triton X-100 could not substitute for DOC. Activity was not detectable when supernate that had been heated at 100°C for 5 min was employed.

The hydrolysis of [^{3}H]myoinositol PI with time is shown in Fig. 4. The dependency of the enzymatic activity upon Ca^{+2} is illustrated in Fig. 5. Simulation by Ca^{+2} was optimal at 5 mM, whereas 10 mM Ca^{+2} was inhibitory. The radioactivity detected in the aqueous supernate was not removable by lyophilization. When chromatographed in two directions on silica paper, <1% of this radioactive material migrated with a lyso-PI standard or with PI. Nearly all of the material was found at the origin. More than 80% of the radiolabeled material in the aqueous supernate migrated with inositol-1-phosphate after descending chromatography, and tested positively for phosphate. The remainder migrated with free myoinositol. Platelet sonicate supernates incubated with [^{3}H]myoinositol did not produce labeled inositol-1-phosphate.

No water-soluble radiolabeled material was produced when [^{14}C]choline PC was substituted for [^{3}H]myoinositol PI as a substrate, irrespective of the pH of the incubation mixture. Further, no DG was produced when sonicate supernates from platelets were incubated with suspensions of PE or PS. Erythrocyte-neutrophil sonicate preparations did not hydrolyze [^{3}H]myoinositol PI to water-soluble material.

Fig. 6 represents the relationship of the hydrolytic activity to the pH of the incubation mixture, displayed in the presence of 2 mg/ml DOC and 5 mM Ca^{+2}. There was a clear optimum at pH 7 for both the generation of water-soluble radioactivity (represented in terms of nanomoles of [^{3}H]myoinositol PI hydrolyzed) and the formation of DG. If one takes the molecular weight of chicken liver microsome PI-derived DG to be 590 (liver PI is considerably less rich in arachidonic acid than is human platelet PI; 26), then it is possible to calculate that the amount of DG produced on a molar basis is comparable both to the amount of [^{3}H]myoinositol PI hydrolyzed and to the amount of water-soluble [^{3}H]myoinositol-containing product formed, with a molar ratio of 0.99±0.14 (SE):1.0.

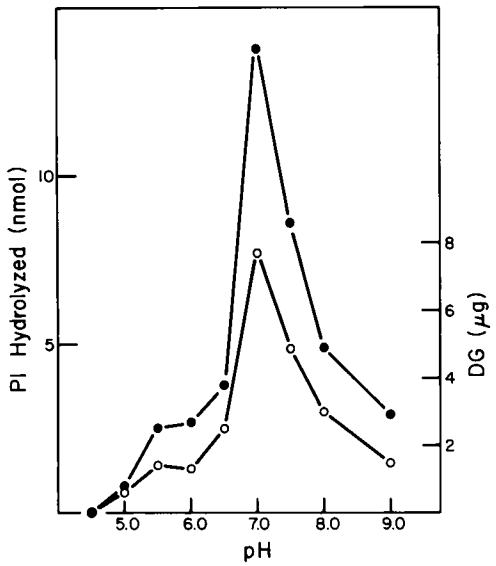

FIGURE 6 Hydrolysis of [^{3}H]myoinositol PI and formation of DG as a function of pH. [^{3}H]Myoinositol PI was incubated with platelet sonicate supernate (1 mg/ml), 2 mg/ml DOC, and 5 mM Ca^{+2} for 60 min at 37°C. The pH was varied by the use of 100 mM acetate-acetic acid and Tris-acetate buffers. The formation of water-soluble [^{3}H]myoinositol was determined as described for Fig. 4. Quantitation of DG in the $CHCl_3$ phase was achieved by densitometry of charred spots on thin-layer plates in comparison with standards. ○, DG; ●, PI.

DISCUSSION

The production of DG appears to be one of the earliest metabolic events to occur during the activation of human platelets by thrombin, anteceding the full secretory response. The identification of DG as such is supported by the presence of alkali-hydrolyzable fatty acid and glycerol, and the observed comigration with known 1,2-DG in two different neutral lipid chromatography systems. Resting platelets contain very small quantities of DG (27). Exposure of platelets to thrombin induces as much as a 30-fold increase in the amount of this material, but the elevation is transitory. Within 2 min, the platelet has essentially achieved the low levels of DG that characterize the resting state.

The source of the DG is most probably PI. Of the other possible candidates for precursor, TG, PA, phospholipid(s) other than PI, and monoglyceride, none is a satisfactory alternative. TG levels do not change significantly during the short time interval of relevance, nor does the content of [^{3}H]arachidonic acid in TG decrease. PA, although difficult to quantitate on a mass basis, does not lose [^{3}H]arachidonic acid with 15 s of exposure to thrombin, but rather, gains this fatty acid. Further, the presence of NaF, which has been shown to inhibit phosphatidate phosphatase (20–21), does not inhibit the production of DG. The formation of DG from PC, PS, or PE would require the action of a phosphatide phosphodiesterase acting upon one or all of these substrates. Thus far, no such activity has been detectable in human platelets (28). Further, PS and diacyl-PE are unchanging with respect to [^{3}H]arachidonic acid and [^{3}H]glycerol in the period examined. PC starts to show significant loss only after 30 s, and continues to do so well after the time that DG has returned to normal levels. Finally, monoglyceride, even in platelets labeled with high specific activity [^{3}H]glycerol, was not found. In contrast, PI loses mass and [^{3}H]arachidonic acid with appropriate rapidity. The specific acitivity of PI with respect to [^{3}H]arachidonic acid is in keeping with that calculated for labeled DG. It is also noteworthy that of all the phospholipids of the platelet, PI displays the highest specific activity when platelets are incubated with [^{3}H]arachidonic acid (8, 10). The specific activity of DG is slightly (15%) greater than that of PI, which in turn has a higher specific activity than PC, PS, and PE. Finally, the existence of a PI phosphodiesterase, described here, could account for the early changes observed.

The data presented in this report constitute direct evidence for a PI-specific phosphodiesterase in human platelets. Three sets of findings support an argument for the specificity of the enzyme for PI. Firstly, the enzyme preparation does not hydrolyze [^{14}C]-choline PC to water-soluble radiolabeled material. Secondly, [^{3}H]myoinositol PI, in the presence of an eightfold excess of PC, a fivefold excess of PE, and an equivalent amount of PS, is hydrolyzed to radiolabeled inositol and DG in equimolar amounts. Were the enzyme acting comparably upon substrates other than PI, one would expect to observe the production of significantly greater amounts of DG than radiolabeled inositol. Finally, the enzyme does not hydrolyze pure suspensions of PS or PE to DG.

It appears that the enzyme is a phospholipase C, producing myoinositol-1-phosphate and DG. Whether or not a myoinositol-1:2-cyclic phosphate is generated in the system, as is the case in certain other cells (21), is unknown because the acidic conditions employed would cause a hydrolysis of the labile cyclic compound to the monoester form.

The existence of platelet PI-phosphodiesterase, and further, its requirement for Ca^{+2} at physiologic pH, are of interest for the elucidation of a secretory mechanism in platelets. The platelet contains large stores of sequestered Ca^{+2} which are apparently mobilized in the cytoplasm during the earliest stages of platelet activation (29–31). Stimulation by a secretion-inducing agent such as thrombin might promote the hydrolysis of PI by making Ca^{+2} available to PI-phosphodiesterase. If the activation of PI-phosphodiesterase is an early

event in the sequence leading to secretion by platelets, as it appears to be, it may provide another control point for platelet functioning and would thus merit investigation in human subjects displaying secretory defects.

The elevation of DG and the release of 5-HT from storage granules are similarly responsive to increased doses of thrombin. Under conditions in which secretion-inducing arachidonic acid oxidation products are not formed, both the generation of DG and secretion in response to thrombin are unimpaired. Thus it appears that the increase in DG is not dependent upon the generation of prostaglandin endo-peroxides, which is blocked by aspirin. However, dBcAMP, which is thought to act, in part, to restrict Ca^{+2} mobilization in platelets (32, 33), effectively inhibits both secretion and DG formation.

The role of DG in the secretory process is as yet unknown. The level of DG has been shown to increase in certain other tissues during secretion (34-37), and Allan and Michell have suggested that DG promotes membrane fusibility in such processes (35). Under certain conditions, DG facilitates fusion of hen erythrocytes (38). Demel et al. (39) reported that the generation of DG in PC monolayers by phospholipase C lowered surface pressures, thereby promoting the breakdown of the monolayers. And recently, Chap et al. (40) observed that the production of diacylglycerols in platelet membranes exposed to *Clostridium welchii* phospholipase C was followed by aggregation and cell lysis.

It is thus possible that localized DG generated in platelet membranes may assist secretion by promoting the fusion of membranes.

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