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S E Rittenhouse, C L Allen

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Research Article

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Synergistic Activation by Collagen and 15-Hydroxy-9α,11α-peroxidoprosta-5,13-dienoic acid (PGH_2) of Phosphatidylinositol Metabolism and Arachidonic Acid Release in Human Platelets

SUSAN E. RITTENHOUSE and CYNTHIA L. ALLEN, *Hematology Division and Hemostasis Unit, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115*

ABSTRACT Collagen stimulates the activation of phosphatidylinositol (PI)-specific phospholipase C (EC 3.1.4.10) in human platelets, as manifested by the disappearance of PI, the transient formation of diacylglycerol (DG), and release of myoinositol. Platelets exposed to collagen also form lysophosphatidylinositol (LPI). Maximum formation of DG occurs within 60 s of the addition of collagen and is in proportion to the concentration of collagen provided, up to 100 μg/2 × 10^9 platelets/ml. Hydrolysis of PI, formation of DG, and release of arachidonic acid are all inhibited ~68% by aspirin or indomethacin, both of which inhibit platelet cyclooxygenase. This inhibition is reversed by the product of cyclooxygenase activity, 15-hydroxy-9α,11α-peroxidoprosta-5,13-dienoic acid (PGH_2), or by the PGH_2 analogue and agonist, U-46619. The counteracting effects of either PGH_2 or the PGH_2 analogue can be blocked, in turn, by a PGH_2 antagonist, U-51605.

Neither PGH_2 nor its stable analogue is, by itself, an efficient stimulus for PI breakdown to DG and LPI in platelets. However, in conjunction with collagen, these agents synergistically promote the net breakdown of PI and the release of arachidonic acid in aspirin-treated platelets. Our findings thereby imply that PGH_2 has an important role in regulating both the release of its precursor, arachidonic acid, and the metabolism of PI induced by collagen.

Dibutyryl cyclic AMP or prostaglandin D_2 (PGD_2), a prostaglandin that elevates concentrations of cAMP in platelets by stimulating adenylate cyclase, inhibits the hydrolysis of PI induced by collagen by 70%. The activation of PI metabolism by collagen appears to be inhibited by cAMP independently of any effects of this inhibitor on the formation of PGH_2.

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INTRODUCTION

Upon exposure to appropriate agonists, several varieties of secretory cells exhibit marked phospholipase C(PLC)[1]-mediated hydrolysis of phosphatidylinositol (PI) resulting in the generation of diacylglycerol (DG) (1). The full nature of the function of such changes in cellular PI and DG is as yet unknown. There are indications that the metabolism of phosphatidylinositides via PLC may be a prerequisite for both the movement of Ca^{+2} within physiologically stimulated cells (1–3) and the release of arachidonic acid and subsequent formation of biologically active 15-hydroxy-9α,11α-peroxidoprosta-5,13-dienoic acid (PGH_2) and thromboxane A_2 (TXA_2) in platelets (4–6). In the platelet, PI-specific PLC can be activated rapidly by thrombin (7), a stimulus for both secretion and the formation of PGH_2 and TXA_2 (8, 9).

Our laboratory has demonstrated recently that the Ca^{+2} ionophore A23187 is an inefficient stimulus for PI-specific PLC, although phospholipase A is activated in platelets exposed to A23187 and TXA_2 is formed (10). This finding implies both that a general, passive flux in Ca^{+2} stores does not activate PLC and that PGH_2/TXA_2, formed in platelets in response to A23187,

[1] *Abbreviations used in this paper*: cAMP, cyclic AMP; ASA, acetylsalicylic acid; DG, diacylglycerol; HHT, 12L-hydroxy-5,8,10-heptadecatrienoic acid; HPLC, high performance liquid chromatography; INDO, indomethacin; LPI, lysophosphatidylinositol; PA, phosphatidic acid; PGH_2, 15-hydroxy-9α,11α-peroxidoprosta-5,13-dienoic acid; PI, phosphatidylinositol; PLC, phospholipase C; TXA_2, thromboxane A_2.

is also a poor stimulus for this enzyme. It was clear from these studies that the factors regulating PI-specific PLC merited further examination.

Numerous investigations of platelet activation have centered upon the role of the potent arachidonic acid metabolites PGH_2 and TXA_2 in inducing aggregation and secretion (for a review, see MacIntyre, 11). However, no studies to date have examined the role of these agonists in controlling PI metabolism and their own production at the level of phospholipase. We have undertaken the present studies to evaluate the effect of PGH_2/TXA_2 upon the hydrolysis of PI and net release of arachidonic acid, and have chosen the physiologic platelet agonist collagen as a costimulus. Collagen is a potent inducer of PGH_2/TXA_2 formation and is particularly relevant to such studies in view of the known ability of cyclooxygenase inhibitors to reduce the prosecretory effects of collagen (12). In this communication, we describe the initial metabolism of PI in platelets exposed to collagen in the presence or absence of several inhibitors of platelet function. We report a synergistic promotion by collagen and PGH_2 of the PI response and arachidonic acid release, thereby implicating PGH_2 in the regulation of phospholipases in platelets.

METHODS

Normal human platelet concentrates were obtained within 15 h of venipuncture and freed of any contaminating erythrocytes and buffy coat (7). In some cases, blood was drawn in the laboratory from normal human volunteers (7). Platelets were incubated in plasma at 37°C for 30 min with [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (New England Nuclear, Boston, MA; 72 Ci/mmol, 50 nM) and washed (7). Some platelet suspensions were incubated as well with 0.56 mM acetylsalicylic acid (ASA) (Sigma Chemical Co., St. Louis, MO) for 30 min before washing. Siliconized glassware was used in all studies.

Labeled platelets (final concentration 2×10^9 cells/ml) were suspended in an albumin-free modified Tyrode's buffer (13) containing 0.8 mM Mg^{+2} and 0.5 mM Ca^{+2} at pH 7, and incubated at 37°C for 0–4 min with 0–150 μg collagen (Hormon-Chemie, Münich, West Germany). Prior to these studies, where appropriate, platelet suspensions were incubated for 15 min with and without freshly prepared ASA (0.56 mM, dissolved in ethanol, final concentration < 0.06%) at room temperature, or for 3 min with indomethacin (INDO) (Sigma Chemical Co., 5.6 μM), or the adenylate cyclase inhibitor 9-(tetrahydro-2-furyl) adenine (SQ22536, 150 μM). SQ22536 was the gift of Dr. D. N. Harris of E. R. Squibb & Sons, Inc., Princeton, NJ. Additional components present in the incubation system at 37°C included: freshly dissolved 0–4 μM PGH_2; 0–4 μM (15S)-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid (U-46619, a PGH_2 analogue); 0–20 μM (5Z,9α,11α,13E)-9,11-azaprosta-5,13-dienoic acid (U-51605); and 0–1 μM PGD_2. PGH_2 was synthesized enzymatically, using [1-^{14}C]arachidonic acid (New England Nuclear) and arachidonic acid (Nu Chek Prep., Inc., Elysian, MN) at a specific activity of 1,000 dpm/nmol, and purified ram seminal vesicle cyclooxygenase (courtesy of Dr. Gerald Roth, University of Connecticut, Farmington, CT), according to the procedure of Gorman et al. (14). Radiolabeling provided a monitor of the quantity of PGH_2 obtained and added in subsequent incubations. The specific activity was low enough so that counting of [^{3}H]arachidonic acid released from platelets was not compromised. The PGH_2 obtained was >95% pure, as determined by thin-layer chromatography on Silica G in an ethyl acetate/acetic acid (99:1, vol/vol) solvent system. Minor impurities were $PGF_{2\alpha}$, PGE_2, and 1% PGD_2. No arachidonic acid was present. [^{14}C]PGH_2 was stored in dry acetone over anhydrous Na_2SO_4 at −20°C. Immediately before use, an aliquot of [^{14}C]PGH_2 was dried under N_2 flow in an ice-cold siliconized glass tube and dissolved in ice-cold 20 mM Tris HCl, pH 7. U-46619 and U-51605 were dissolved in ethanol (final concentration < 0.3%). This concentration of ethanol had no detectable effect in our studies. In some experiments, platelets were incubated with 2 U/ml purified human α-thrombin (1 nM = 0.11 U/ml, kindly provided by Dr. J. W. Fenton, II, New York State Dept. of Health) for 15 s and 2 min, in the presence and absence of 10 μM U-51605.

Incubations were terminated by the addition of 3.75 vol chloroform/methanol (1:2), and lipids were extracted by a modified Bligh and Dyer (10) procedure. Extracts were divided, and neutral lipids and phospholipids were resolved and quantitated as described previously (7, 10). The method of Van Rollins et al. (15) was used for the resolution of cyclooxygenase metabolites by high performance liquid chromatography (HPLC), using a 4.6 × 250-mm Ultrasphere ODS C-18 reverse-phase column (Altex Scientific Inc., Berkeley, CA), a Beckman model 110A pump (Beckman Instruments, Inc., Fullerton, CA), and a Perkin-Elmer LC-75 variable wavelength spectrophotometer (Perkin-Elmer Corp., Norwalk, CT), monitoring at 205 nm. The resolved metabolites were compared with standards kindly provided by Dr. John Pike (Upjohn Co., Kalamazoo, MI), who also supplied U-46619 and U-51605.

Data obtained after HPLC indicated that the methanol/H_2O fraction resulting from the Bligh and Dyer procedure contained all of the TXB_2 (the stable metabolite of TXA_2) produced during the incubations. Therefore, suitable aliquots of this fraction were diluted and used for determinations of TXB_2 content by standard radioimmunoassay (RIA) (New England Nuclear TXB_2 ^{3}H RIA Kit). The RIA used a 1:2,000 dilution of TXB_2 antibody (Seragen Inc., Boston, MA), 0–3 pmol TXB_2, 10,000 dpm [^{3}H]TXB_2 (125 Ci/mmol), and a phosphate-gelatin buffer. After an incubation at 4°C overnight, charcoal-coated dextran was used to separate antibody-bound and unbound radioactivity, which was quantitated by scintillation spectrophotometry (10).

In addition to the above, extraction and chromatographic procedures were designed for optimal quantitation of PI, lysophosphatidylinositol (LPI), phosphatidic acid (PA), and myoinositol. Efficiencies of recovery were determined after extraction of [^{3}H]myoinositol-PI, [^{3}H]myoinositol-LPI, [2-^{3}H(*N*)]myoinositol, or [^{14}C]oleoyl PA, which were added to preparations of unlabeled platelets. [^{14}C]PA was derived from [^{14}C]oleoyl phosphatidylcholine (New England Nuclear) by digestion with cabbage leaf phospholipase D (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA; 16) from which the yield was 95%. [^{3}H]Myoinositol-LPI was generated from the corresponding [^{3}H]PI (17) by digestion for 60 min at 37°C with bee venom phospholipase A_2 (Sigma Chemical Co.) in a 200 μl mixture containing 0.4 mg enzyme, 0.15 mg [^{3}H]PI, 5 mM $CaCl_2$, and 100 mM glycine-NaOH, pH 8.5, and extraction with chloroform/methanol and *n*-butanol, as described below. The yield of [^{3}H]LPI was 90%.

Washed human platelets (2.5×10^9/ml), labeled or unlabeled with [^{3}H]arachidonic acid, were incubated as previously described, in a final volume of 2.0 ml. Incubations were terminated with 7.5 ml ice-cold chloroform/methanol (1:2) containing 50 μl of 80 mM Na_2 EDTA, pH 7. Mixtures were kept in an ice-water bath for 30 min, after which 2.0 ml ice-cold chloroform was added, with mixing. Phases were separated after centrifugation at 1,000 *g* for 15 min at 4°C. The lower phase was removed to concentrator vials, and the upper phase was extracted with 2.5 ml cold chloroform and spun as above. The lower phase was pooled with that in the concentrator vial. Samples (20 μl) of the upper aqueous phase were then taken for analysis of TXB_2. The upper phase (~3 ml) was then evaporated under N_2 flow to 1.0 ml and diluted to 1.5 ml with H_2O. This solution was extracted with 750 μl *n*-butanol (99 mol % pure, Fisher Scientific Co., Pittsburgh, PA) and spun as above. The upper *n*-butanol phase was pooled with the chloroform extracts in the concentrator vials and evaporated under N_2. These extracts contained 97.0% (SEM 1.2, $n = 4$) of added [^{3}H]PI, [^{3}H]LPI, and [^{14}C]PA. The aqueous phase resulting from the extraction with *n*-butanol contained 89.1% (SEM 1.0, $n = 4$) of added [^{3}H]myoinositol and was saved. The combined extracts were dissolved in chloroform/methanol (1:1) and applied to 0.7-cm o.d. columns containing 0.30 ml neomycin coupled to glass beads. The neomycin-coupled matrix was prepared according to the method of Schacht (18), and columns were washed as described by Palmer (19). All neutral lipids and nonacidic phospholipids and lysophospholipids came through the columns in fractions 1 and 2 (19). Elution of acid phospholipids was achieved with a modification of Palmer's scheme (19): PA was collected in vials containing 10 μl 8.5 M NH_4 formate after being eluted from the columns with 6 vol each of chloroform/methanol/88% formic acid (5:10:1) and (5:10:2), and 2 vol of chloroform/methanol/H_2O (5:10:2). Eluates, containing 95% of added [^{14}C]PA, were concentrated under N_2 and applied to unactivated Whatman LK-5 (250 μm) chromatography plates (Whatman Inc., Clifton, NJ). PA ($R_f = 0.83$) was separated from phosphatidylserine ($R_f = 0.66$) by development with chloroform/methanol/diisobutylketone (DIBK)/acetic acid/H_2O (79:26:53:35:6). Two-dimensional chromatography of this fraction on silica-impregnated paper (20) confirmed that only PA and phosphatidylserine were present in this fraction. Lipids were detected under UV light with rhodamine spray (0.003%) and PA was eluted, successively, with 3 ml chloroform/methanol/2N HCl (25:15:2.5), 1 ml methanol, and 1 ml methanol/H_2O (95:5). Eluates were assayed for phosphorus by the method of Duck-Chong (21). All glassware and reagents were scrupulously prepared to minimize phosphorus contamination.

PI, LPI, cardiolipin, and cerebroside sulfate were eluted from neomycin columns with 6 vol chloroform/methanol/340 mM NH_4 formate (5:10:2) and resolved on unactivated LK-5 plates with chloroform/methanol/DIBK/pyridine/0.5 M NH_4Cl, pH 10.4 (30:17.5:25:35:6) solvent. LPI ($R_f = 0.11$) and PI ($R_f = 0.24$) were well separated from each other and cardiolipin + cerebroside sulfate ($R_f = 0.48$). The composition of this fraction, which contained 97% of added [^{3}H]PI and [^{3}H]LPI, was confirmed by two-dimensional chromatography (20). PI-4-phosphate and PI-4,5-bisphosphate were eluted from the columns only with much higher concentrations of NH_4 formate, as noted by Palmer (19) and Schacht (18). After visualization, PI and LPI were eluted from silica gel with the same solvent system used to elute PA but for the substitution of H_2O for HCl. Phosphorus was quantitated as noted above (21). In addition, where platelets labeled with [^{3}H]arachidonic acid were used, the content of ^{3}H in PI, PA, and LPI was determined and the specific activities compared.

The aqueous phase saved from the *n*-butanol extraction was digested first with 6 N HCl, as described by Bell and Majerus (22), or applied directly to a 0.5 g mixed-bed resin (AG50W-X8 + AG 1-X8, Bio-Rad Laboratories, Richmond, CA) and eluted completely in 4.0 ml H_2O. The eluate was lyophilized and the trimethylsilyl derivative was formed by incubation for 30 min with 400 μl of a mixture of TriSil concentrate and pyridine (3:10; Pierce Chemical Co., Rockford, IL). Solvent was removed under dry N_2 flow and the derivative dissolved in *n*-hexane (HPLC grade, Fisher Scientific Co.). A portion of this solution was counted for [^{3}H]myoinositol to determine the efficiency of derivatization and recovery (96%). The content of myoinositol was determined in comparison with myoinositol standards, using a gas chromatograph (model 5840A, Hewlett-Packard Co., Palo Alto, CA) fitted with a 3% OV-1 column, flame ionization detector, reporting integrator, and programmable oven, which varied the column temperature from 140 to 200°C at the rate of 2°/min. Retention time for trimethyl-silyl-myoinositol, which was well separated from other carbohydrates, including glucose anomers, was 28.5 min (flow rate 20 ml/min).

Adenylate cyclase activity in human platelet membranes was assayed in the presence and absence of 1 μM PGD_2 and 0–150 μM SQ22536 by a procedure described by Alexander et al. (23), a modification of the method of Salomon et al. (24). The assay utilized [α-^{32}P]ATP as substrate and directly measures the [α-^{32}P]cAMP produced. PGD_2 has been shown to stimulate platelet adenylate cyclase (25, 26).

RESULTS

As shown in Fig. 1A, hydrolysis of PI and formation of DG occurred in parallel in response to varied concentrations of collagen. Maximum changes were found in the presence of 100 μg/ml collagen/2×10^9 platelets/ml. As is the case when thrombin is the stimulus (7), triglyceride levels were unaltered upon exposure of platelets to collagen. Platelets achieved a maximum net hydrolysis of PI 2 min after exposure to collagen (Fig. 1B). This degree of hydrolysis was equal to ~8 nmol/2×10^9 platelets, or 25% of total PI. Release of arachidonic acid followed a similar pattern (Figs. 2A and B) but exceeded the loss of PI. A transient formation of DG occurred within this period (Fig. 1B). DG reached its peak concentration within 60 s, accounting for 10–15% of the PI hydrolyzed, approximately the percentage that accumulates in response to thrombin (7, 10). Both the dose-response and kinetic data presented are consistent with the findings of Broekman et al. (27), monitoring PI hydrolysis under comparable conditions. In response to a 60-s exposure to 100 μg/ml collagen, platelets formed 1.7 nmol $TXB_2/2 \times 10^9$ platelets, and an equivalent amount of 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), as gauged by radioactivity (Table I); both are cyclooxygenase products originating from PGH_2. Negligible $PGF_{2\alpha}$, PGE_2, or PGD_2 was formed. ASA or INDO

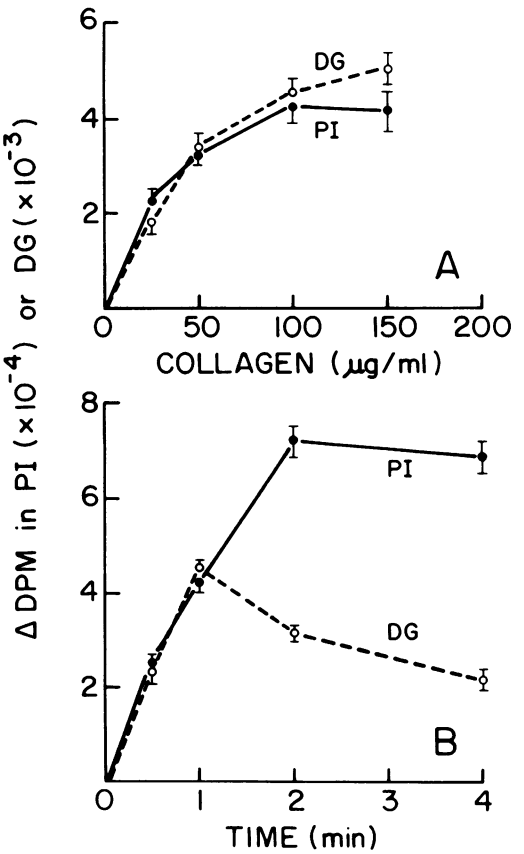

FIGURE 1 Hydrolysis of PI and formation of DG in platelets exposed to collagen. Washed platelets (2×10^9/ml) containing esterified [^{3}H]arachidonic acid were incubated with varied concentrations of collagen for 60 s (A) or 100 μg/ml of collagen for varied periods (B). The gain of radiolabel in DG vs. the loss in PI was determined. Results are shown as the mean±SEM for five experiments performed with the platelets of separate donors.

inhibited >99% of HHT and TXB_2 formation, whereas U-51605, a known inhibitor of thromboxane synthetase, blocked synthesis of TXB_2 and enhanced the conversion of PGH_2 to $PGF_{2\alpha}$ and PGE_2 as indicated in Table I. PGD_2 concentrations were not comparably altered. The addition of [^{14}C]PGH_2 to aspirin-treated platelets yielded equivalent amounts of [^{14}C]TXB_2 and [^{14}C]HHT, reaching a maximum within 30 s of addition. U-46619 migrated as a single band when chromatographed (alone or after incubation with aspirin-treated platelets) in the ethyl acetate/acetic acid solvent system described and led to no formation of TXB_2 in platelets. Thus, it contained no PGH_2 and was not metabolized detectably. Measurement of TXB_2 (and equivalent HHT) provided a useful monitor of the minimum amount of PGH_2 which had reached and been metabolized by the platelets.

ASA and INDO each inhibited the collagen-induced hydrolysis of PI to DG by a mean of 68% (Fig. 3A) under conditions in which prostaglandin formation was blocked >99%. The net release of arachidonic acid was inhibited in parallel with the metabolism of PI and DG.

These findings are in contrast to those obtained with thrombin as a stimulus (5, 7, 10). PGD_2 (1 μM) inhibited TXB_2 formation, arachidonic acid release, and PI metabolism (Fig. 3A) by 70%. Approximately 90% inhibition occurred when aspirin-treated platelets were exposed to PGD_2. This was also the case when dibutyryl cAMP (1 mM) was substituted for PGD_2, but not

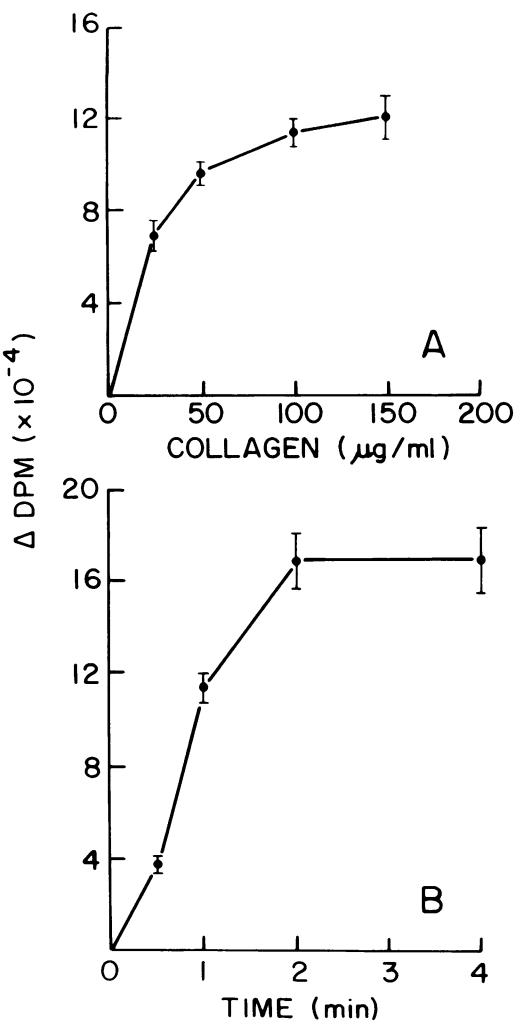

FIGURE 2 Liberation of arachidonic acid in response to collagen. Platelets were incubated as in Fig. 1 with different concentrations of collagen (A) or for varied periods (B). Results are expressed as the mean±SEM for five experiments. "Arachidonic acid" designates the sum of arachidonic acid plus oxygenated metabolites.

TABLE I

*Effect of Inhibitors upon Cyclooxygenase and Thromboxane Synthetase Products in Platelets Stimulated by Collagen**

	TXB_2		HHT		$PGF_{2\alpha}$		PGE_2		PGD_2	
Inhibitor	I	II	I	II	I	II	I	II	I	II
None	7,040	5,195	7,120	5,310	253	195	108	72	38	30
ASA	60	45	53	48	22	25	21	23	30	28
U-51605	291	225	330	286	2,856	1,978	1,428	1,000	48	42

* Platelets (2×10^9/ml) labeled with [^{3}H]arachidonic acid were incubated for 60 s with collagen (100 μg/ml) in the presence of U-51605 (10 μM) or after incubation with ASA (0.56 mM). Labeled products of arachidonic acid metabolism were resolved by HPLC and quantitated by scintillation spectrography. The values (dpm) for platelets from two different donors are shown.

if 150 μM SQ22536, an adenylate cyclase inhibitor, was added in the presence of PGD_2 (data not shown). Measurements of adenylate cyclase activity in platelet membranes revealed that 50 μM and 150 μM SQ22536 inhibited PGD_2-stimulated activity by 50 and 95%, respectively. These results are in general agreement with those of Salzman (28), working with intact platelets.

It was possible to overcome the inhibitory effects of ASA upon the net breakdown of PI and release of arachidonic acid by the addition of either 2 μM PGH_2 or U-46619, an agonist and analogue of PGH_2 not convertible to TXA_2. This was the case whether ASA was present during the incubation or had been preincubated with platelets and the excess ASA washed away. The effect of either PGH_2 or its analogue could be blocked by 10 μM U-51605 (Fig. 3B). U-51605 inhibits thromboxane synthetase (29); however, U-51605 clearly antagonizes the effects of the PGH_2 analogue, as well. This finding is consistent with that of MacIntyre (11). U-51605 does not inhibit phospholipase directly, since we have observed that it does not block PI metabolism and arachidonic acid release stimulated by thrombin. Formation of DG in the presence of U-51605 and thrombin was 95–98% of that for control platelets. Hydrolysis of PI and net release of arachidonic acid + metabolites were similarly unaffected, whereas formation of TXB_2 was inhibited 98%.

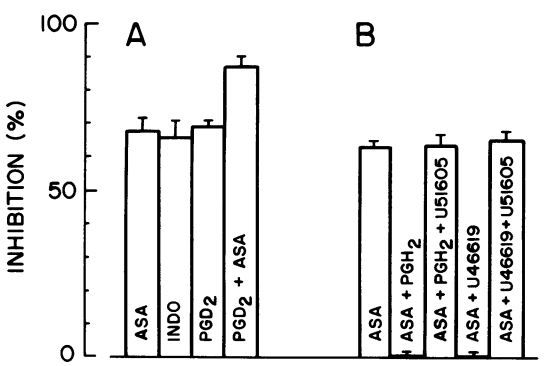

FIGURE 3 Inhibition of collagen-induced changes in lipid pools under various conditions. Platelets were incubated for 60 s with 100 μg/ml collagen at 37°C. Where indicated, other agents were present at the following concentrations: ASA, 0.56 mM or preincubated with platelets at this concentration and then removed by washing; INDO, 5.6 μM; PGH_2, 2 μM; PGH_2 analogue U-46619, 2 μM; PGH_2 antagonist U-51605, 10 μM; PGD_2, 1 μM. Inhibition of PI hydrolysis, DG formation, and net release of arachidonic acid + metabolites was calculated and found to be the same for these three lipid classes. Results shown in A are the means±SEM for experiments performed with the platelets from 10 different donors (ASA, INDO) or 3 different donors (PGD_2). Results in B are the means±SEM for platelets from 4 different donors. Data are expressed vs. inhibitor-free controls containing buffer±0.3% ethanol (ethanol was without effect).

Fig. 4 illustrates the time course of PI and DG metabolism for platelets exposed to collagen in the presence and absence of ASA and 2 μM PGH_2. ASA inhibited the PI response after both 60 and 120 s. The addition of PGH_2 led to the normal metabolism of these two species, which was unaffected by the presence of 150 μM SQ22536. Since SQ22536 blocks the elevation of platelet cAMP by PGD_2, it is clear that the slight contamination of PGH_2 by PGD_2 did not interfere with the platelet response to PGH_2.

As shown in Table II, stimulation of platelets for 2 min with collagen led to the formation of LPI, accounting for ~25% of the loss in PI. This species contained no [^{3}H]arachidonic acid. Free myoinositol accounted for the majority of the change in PI. As has been observed to be the case for thrombin-stimulated platelets (22), unhydrolyzed extracts accounted for most of the free myoinositol, implying that most of the myoinositol phosphate arising from the action of PLC had been digested by platelet phosphatases. PA, presumably derived via phosphorylation of DG, was

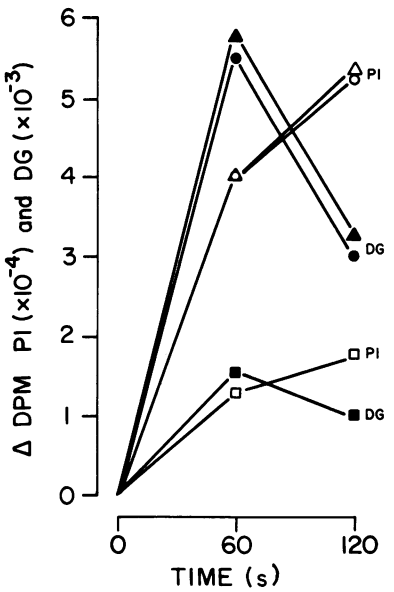

FIGURE 4 Effect of ASA or ASA + PGH_2 on collagen-induced PI hydrolysis and DG formation with time. Labeled platelets (Fig. 1) were incubated with collagen (100 μg/ml, ●); collagen + ASA (100 μg/ml, ■); or collagen + ASA + PGH_2 (2 μM, ▲).

observed in platelets exposed to collagen, as has been reported by Broekman et al. (27). Treatment of platelets with aspirin before incubation with collagen inhibited the loss of PI, as we have noted already, as well as the formation of LPI, PA, and myoinositol. The addition of PGH_2 to such platelets (resulting in the formation of 0.3 nmol TXB_2/2.5 × 10^9 platelets) overcame much of the aspirin-induced inhibition. In all cases (data not shown), the specific activities of PI and PA were equivalent and did not themselves vary with incubation conditions. PGH_2 added in the absence of collagen, but yielding an equal amount of TXB_2 ±collagen, induced very little hydrolysis of PI and formation of PA, LPI, and myoinositol. However, PGH_2/TXA_2 had a potentiating effect upon the stimulation of platelet lipid metabolism by collagen, an observation which is also represented in Fig. 5.

The formation of DG and release of arachidonic acid induced in aspirin-treated platelets exposed for 60 s to collagen were markedly enhanced by PGH_2, as shown in Fig. 5, upper curve. As an index of the access of platelets to PGH_2 in the presence and absence of collagen, the formation of TXB_2 was monitored by RIA, shown on the abscissa. PGH_2 alone (lower curve) did not strongly promote the formation of DG or free arachidonic acid. This was also the case when varied concentrations of U-46619 were substituted for PGH_2. The addition of PGH_2 to collagen-exposed platelets incubated with aspirin (upper curve) led to significantly more formation of DG and free arachidonic acid than did the theoretical sum of collagen + ASA and PGH_2 + ASA. The synergistic enhancement by collagen (100 μg/ml) and PGH_2 (1 μM) of DG production, PI hydrolysis, and the release of free arachidonic acid ranged in five experiments from 180–300% of the sum of the separate values. Similar, if not quite as marked, findings of synergism were attained with U-46619. These effects were unaltered by the addition of PGE_2 or $PGF_{2\alpha}$ at the concentrations found in preparations of PGH_2. As little as 0.8 μM PGH_2/2 × 10^9 platelets was sufficient both to achieve a maximum synergistic effect and to overcome the inhibitory effects of ASA on collagen-initiated PI metabolism. This

TABLE II
Changes in the Content of PI and Its Metabolites in Stimulated Human Platelets

Stimulus	Δ (nanomoles)			
	PI	LPI	PA	Myoinositol
Collagen	−23.5±0.6	5.9±0.8	6.6±0.5	16.0±1.5
Collagen + ASA	−7.6±0.6	1.9±0.6	1.2±0.5	4.5±1.0
Collagen + ASA + PGH_2	−16.0±0.7	4.2±0.3	4.8±0.7	11.5±1.2
ASA + PGH_2	−1.4±1.0	0.2±0.2	0.5±0.2	1.0±0.9

* 2 ml of washed human platelets (2.5 × 10^9/ml, containing 94 nmol PI) were incubated as indicated above with and without collagen (100 μg/ml) in the presence or absence of ASA (0.56 mM) and PGH_2 (yielding 0.3 nmol TXB_2/ml) for 120 s. Incubations were terminated with chloroform/methanol (1:2) and the lipids and myoinositol extracted and quantitated as described in the text. Results were compared with those for platelets incubated with buffer±ASA (these did not differ) and are expressed as the mean change±SEM for four experiments performed in duplicate with the platelets from different donors.

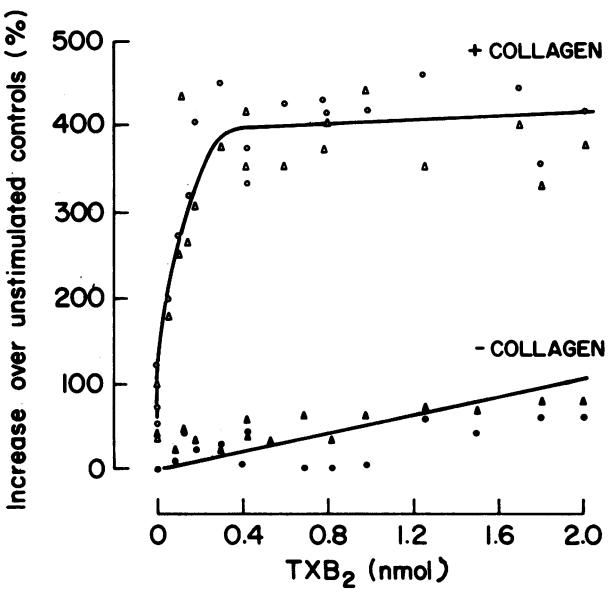

FIGURE 5 Stimulation of the release of DG and arachidonic acid by PGH_2 in the presence and absence of collagen. The amount of PGH_2 added to 2×10^9 platelets is expressed on the abscissa in terms of the amount of TXB_2 formed. Platelets had been exposed to ASA (0.56 mM) before the addition of other agents and were incubated for 60 s at 37°C with collagen (100 μg/ml, upper curve) or without collagen (lower curve). The increases in the release of DG (Δ, ▲) and arachidonic acid + metabolites (O, ●) were determined with respect to platelets incubated with buffer alone and are presented for experiments conducted with the platelets of five different donors.

amount is only 24% of the quantity of PGH_2 (=TXB_2 + HHT) produced by 2×10^9 platelets responding to 100 μg/ml of collagen.

DISCUSSION

Four major conclusions can be reached based on our data:

First, PGH_2 synergistically enhances the metabolism of PI and the net release of arachidonic acid from PI and other phospholipids in platelets exposed to collagen. PLC is responsible for the majority of the hydrolysis of PI observed. However, we have also detected LPI in stimulated platelets, accounting for ~25% of the decrease in PI. The formation of this species in platelets exposed to thrombin and ionophore has been reported recently (30). LPI could be formed directly by the action of phospholipase A_2, an activity which had been indicated by previous findings in our laboratory (10). It is conceivable, however, that LPI could be formed by a more circuitous route:

$$PA \xrightarrow{PLA_2} \text{lysoPA} \xrightarrow{CTP} \text{CDP} \cdot \text{monoglyceride} + PP_i \xrightarrow{\text{myoinositol}} \text{LPI} + \text{CMP}$$

The presence in platelets of a phospholipase A_2 that is active against PA, leading to the formation of lysoPA, has been documented (6). However, formation of cytidine diphosphate (CDP) monoglyceride, or transfer of myoinositol to such an intermediate in platelet extracts, has not yet been reported. Should the second route occur in platelets, the formation of LPI would be dependent upon a variety of enzymes, including PI-specific PLC. In either case, the formation of LPI, like the release of arachidonic acid and the formation of the metabolites clearly arising from the action of PI-specific PLC, is inhibited by aspirin and synergistically potentiated by PGH_2. The means by which PGH_2 achieves these effects is an intriguing subject for speculation. We cannot rule out the possibility that PGH_2 increases the binding of platelets to collagen in our system. However, PGH_2 may act more directly to enhance the activity of phospholipases, once the enzymes have been "primed" by the binding of the platelet to collagen. In this regard, it would be useful to know whether PGH_2 promotes the "PI effect" of other cell types responding to appropriate agonists.

Second, the results for experiments with the PGH_2 analogue and platelet agonist U-46619 imply that PGH_2 may act synergistically in the absence of TXA_2, although it is difficult at this stage to assess the extent to which TXA_2 may participate. It is possible, for example, that TXA_2, PGH_2, U-46619, and U-51605 can all compete for the same receptor. Whereas U-46619 does not appear to be metabolized in short periods of incubation, PGH_2 can be transformed rapidly to PGE_2 and $PGF_{2\alpha}$ when thromboxane synthetase is inhibited. Thus, it is not clear whether sufficient PGH_2 could accumulate in the absence of thromboxane formation to produce the effects obtainable with U-46619.

Third, PGH_2 does not strongly promote PI metabolism and arachidonate liberation, except in conjunction with collagen. Our findings are consistent with the observations of Diminno et al. (31), who have noted that, whereas the PGH_2 analogue U-46619 (0.9–12 μM) induces platelet aggregation, it does not lead to prostaglandin production. We have found that arachidonic acid is not released in response to U-46619 or PGH_2. Neither PI-specific PLC nor phospholipase A_2 is activated under these conditions.

Finally, elevation of the platelet's content of cAMP effectively blocks PI metabolism and arachidonic acid liberation, including that portion which is not dependent upon PGH_2 formation. PGD_2 inhibits the hydrolysis of PI, the release of arachidonic acid, and the formation of TXB_2/HHT. PGD_2 does not appear to achieve this general inhibition by depressing cyclooxygenase activity, since the response of aspirin-treated platelets to collagen is inhibited 90% by PGD_2. The findings with PGD_2 and dibutyryl cAMP for platelets

responding to collagen are in keeping with results obtained with thrombin as a stimulus (32–34). The PI response in thrombin-stimulated platelets (which is not dependent on cyclooxygenase activity) is also inhibited by cAMP (7). Thus, whether collagen or thrombin is the stimulus, cAMP exerts its major effects before prostaglandin formation.

The major findings reported here have some interesting clinical and pathophysiological implications. We have observed that platelets responding to collagen normally produce four to five times more PGH_2 than they need as a synergistic cohort. Thus, platelets with below normal cyclooxygenase activity due either to partial inactivation or deficiency of this enzyme should nonetheless display a normal PI response and mobilize arachidonic acid when exposed to collagen, provided that they can synthesize at least 0.8 μM $PGH_2/2 \times 10^9$ cells. The role of PGH_2 as a potent synergistic promoter, but not initiator, of PI breakdown and arachidonate release implies that platelets interacting with the intima of vessel walls should release the most arachidonic acid and form the most prostaglandin where it is needed, at the site of injury. Yet, the PGH_2 and TXA_2 diffusing to platelets more distant from exposed intima should not lead endlessly to more prostaglandin formation.

It is clear from this and our previous study (10) that not all platelet agonists activate PLC. The role fulfilled by the hydrolysis of PI to DG in platelets responding to thrombin or collagen may be duplicated by the Ca^{+2} ionophore A23187 or by PGH_2, thereby obviating the need for PLC activation to achieve the secretory response. Such a role could be the promotion of a flux in Ca^{+2}. Michell (1, 2) has noted that the hydrolysis of PI by PLC is directly coupled in many cell types to receptor stimulation and has proposed that this event leads to the transport of Ca^{+2}. PI-PLC in this model would have a Ca^{+2}-gating function. If PI-PLC activation is required for movement of Ca^{+2} to occur, it is reasonable to expect that a Ca^{+2} flux should not of itself activate PI-PLC. PI-PLC is indeed dependent upon Ca^{+2} (7, 35), but there may well be sufficient membrane-bound Ca^{+2} available in quiescent platelets to permit the initial activity of PI-PLC that occurs in response to collagen. However, amplification by PGH_2 of the initial "receptor"-activated breakdown of PI by PLC might still take place. Cyclic AMP may act to interfere with the coupling of platelet PI-PLC to the thrombin and collagen "receptors," or to promote the resynthesis of PI, or both.

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REFERENCES

1. Michell, R. H. 1975. Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta.* **415:** 81–147.
2. Michell, R. H., and C. J. Kirk. 1981. Why is phosphatidylinositol degraded in response to stimulation of certain receptors? *Trends Pharmacol. Sci.* **2:** 86–89.
3. Gerrard, J. M., D. A. Peterson, and J. G. White. 1981. Calcium mobilization. *In* Platelets in Biology and Pathology. J. L. Gordon, editor. Elsevier/North Holland, Amsterdam. **2:** 407–436.
4. Bell, R. L., D. A. Kennerly, N. Stanford, and P. W. Majerus. 1979. Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proc. Natl. Acad. Sci. USA.* **76:** 3238–3241.
5. Rittenhouse-Simmons, S. 1980. Indomethacin-induced accumulation of diglyceride in activated platelets: the role of diglyceride lipase. *J. Biol. Chem.* **255:** 2259–2262.
6. Billah, M. M., E. G. Lapetina, and P. Cuatrecasas. 1981. Phospholipase A_2 activity specific for phosphatidic acid: a possible mechanism for the production of arachidonic acid in platelets. *J. Biol. Chem.* **256:** 5399–5403.
7. Rittenhouse-Simmons, S. 1979. Production of diglyceride from phosphatidylinositol in activated human platelets. *J. Clin. Invest.* **63:** 580–587.
8. Hamberg, M., J. Svensson, and B. Samuelsson. 1974. Prostaglandin endoperoxides. A new concept concerning the mode of action and release of prostaglandins. *Proc. Natl. Acad. Sci. USA.* **71:** 3824–3828.
9. Hamberg, M., J. Svensson, and B. Samuelsson. 1975. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. USA.* **72:** 2994–2998.
10. Rittenhouse-Simmons, S. 1981. Differential activation of platelet phospholipases by thrombin and ionophore A23187. *J. Biol. Chem.* **256:** 4153–4155.
11. MacIntyre, D. E. 1981. Platelet prostaglandin receptors. *In* Platelets in Biology and Pathology. J. L. Gordon, editor. Elsevier/North Holland, Amsterdam. **2:** 211–247.
12. Malmsten, C., M. Hamberg, J. Svensson, and B. Samuelsson. 1975. Physiological role of an endoperoxide in human platelets: hemostatic defect due to platelet cyclooxygenase deficiency. *Proc. Natl. Acad. Sc. USA.* **72:** 1446–1450.
13. Rittenhouse-Simmons, S., and D. Deykin. 1976. Isolation of membranes from normal and thrombin-treated gel-filtered platelets using a lectin marker. *Biochim. Biophys. Acta.* **426:** 688–696.
14. Gorman, R. R., F. F. Sun, O. V. Miller, and R. A. Johnson. 1977. Prostaglandins H_1 and H_2: convenient biochemical synthesis and isolation. Further biological and spectroscopic characterization. *Prostaglandins.* **13:** 1043–1053.
15. Van Rollins, M., S. H. K. Ho, J. E. Greenwald, M. Alexander, N. J. Dorman, L. K. Wong, and L. A. Horrocks. 1980. Complete separation by high performance liquid chromatography of metabolites of arachidonic acid from

incubation with human and rabbit platelets. *Prostaglandins.* **20:** 571–577.
16. Eibl, H., and S. Kovatchev. 1981. Preparation of phospholipids and their analogues by phospholipase D. *In* Methods in Enzymology. J. M. Lowenstein, editor. Academic Press, Inc., New York. **72:** 632–639.
17. Rittenhouse, S. E. 1982. Preparation of selectively-labeled phosphatidylinositol and assay of phosphatidylinositol-specific phospholipase C. *In* Methods in Enzymology. W. E. M. Lands and W. L. Smith, editors. Academic Press, Inc., New York. **86:** 3–11.
18. Schacht, J. 1978. Purification of polyphosphoinositides by chromatography on immobilized neomycin. *J. Lipid Res.* **19:** 1063–1067.
19. Palmer, F. B. St. C. 1981. Chromatography of acid phospholipids on immobilized neomycin. *J. Lipid Res.* **22:** 1296–1300.
20. Wuthier, R. E. 1966. Two-dimensional chromatography on silica gel-loaded paper for the microanalysis of polar lipids. *J. Lipid Res.* **7:** 544–550.
21. Duck-Chong, C. G. 1979. A rapid sensitive method for determining phospholipid phosphorus involving digestion with magnesium nitrate. *Lipids.* **14:** 492–497.
22. Bell, R. L., and P. W. Majerus. 1980. Thrombin-induced hydrolysis of phosphatidylinositol in human platelets. *J. Biol. Chem.* **255:** 1790–1792.
23. Alexander, R. W., B. Cooper, and R. I. Handin. 1978. Characterization of the human platelet α-adrenergic receptor. Correlation of [^{3}H]dihydroergocryptine binding with aggregation and adenylate cyclase inhibition. *J. Clin. Invest.* **61:** 1136–1144.
24. Salomon, Y., C. Londos, and M. Rodbell. 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58:** 541–548.
25. Tateson, J. E., S. Moncada, and J. R. Vane. 1977. Effects of prostacyclin on cyclic AMP concentrations in human platelets. *Prostaglandins.* **13:** 389–397.
26. Miller, O. V., and R. R. Gorman. 1979. Evidence of distinct prostaglandin I_2 and D_2 receptors in human platelets. *J. Pharmacol. Exp. Ther.* **210:** 134–140.
27. Broekman, M. J., J. W. Ward, and A. J. Marcus. 1980. Phospholipid metabolism in stimulated human platelets: changes in PI, PA, and lysophospholipids. *J. Clin. Invest.* **66:** 275–283.
28. Salzman, E. W. 1977. Interrelation of prostaglandin endoperoxide and cyclic 3,5′-adenosine monophosphate in human blood platelets. *Biochim. Biophys. Acta.* **499:** 48–60.
29. Gorman, R. R. 1980. Biochemical and pharmacological evaluation of thromboxane synthetase inhibitors. *In* Advances in Prostaglandin and Thromboxane Research. B. Samuelsson, P. W. Ramwell, and R. Paoletti, editors. Raven Press, New York. **6:** 417–425.
30. Billah, M. M., and E. G. Lapetina. 1982. Formation of lysophosphatidylinositol in platelets stimulated with thrombin or ionophore A23187. *J. Biol. Chem.* **257:** 5196–5200.
31. Diminno, G., V. Bertele, L. Bianchi, B. Barbieri, C. Cerletti, E. Dejana, G. Degaetano, and M. J. Silver. 1981. Effects of an epoxymethane stable analogue of prostaglandin endoperoxides (U-46619) on human platelets. *Thromb. Haemostasis.* **45:** 103–106.
32. Gerrard, J. M., J. D. Peller, T. P. Krick, and J. G. White. 1977. Cyclic AMP and platelet prostaglandin synthesis. *Prostaglandins.* **14:** 39–50.
33. Minkes, M., N. Stanford, M. M-Y. Chi, G. J. Roth, A. Raz, P. Needleman, and P. W. Majerus. 1977. Cyclic adenosine 3,5′-monophosphate inhibits the availability of arachidonate to prostaglandin synthetase in human platelet suspensions. *J. Clin. Invest.* **59:** 440–454.
34. Rittenhouse-Simmons, S., and D. Deykin. 1978. The activation by Ca^{+2} of platelet phospholipase A: effects of dibutyryl cyclic adenosine monophosphate and 8-(*N*,*N*-diethyl-amino)-octyl-3,4,5-trimethoxybenzoate. *Biochim. Biophys. Acta.* **543:** 409–422.
35. Mauco, G., H. Chap, and L. Douste-Blazy. 1979. Characterization and properties of a PI phosphodiesterase (PLC) from platelet cytosol. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **100:** 367–370.