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Arachidonate metabolism, 5-hydroxytryptamine release and aggregation in human platelets activated by palmitaldehyde acetal phosphatidic acid

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1 Palmitaldehyde acetal phosphatidic acid (PGAP) caused dose-dependent aggregation of human platelets resuspended in modified Tyrode medium, with a threshold concentration of 0.5–1 μM and an EC_{50} of 4 μM. Concentrations of PGAP which elicited biphasic irreversible aggregation concomitantly induced formation of 1.02 ± 0.029 nmol (mean ± s.e.mean) of malondialdehyde (MDA) per 10^9 platelets and caused release of 58 ± 2.8% of platelet [^{14}C]-5-hydroxytryptamine ([^{14}C]-5-HT) from prelabelled platelets; no MDA formation or [^{14}C]-5-HT release occurred at lower doses of PGAP which elicited only monophasic reversible aggregation. Adenosine 5′-pyrophosphate (ADP)-induced platelet activation resulted in formation of 0.344 ± 0.004 nmol of MDA per 10^9 platelets in association with irreversible aggregation and 49.1 ± 1% release of [^{14}C]-5-HT.

2 Mepacrine, a phospholipase A_2 inhibitor, at 2.5 μM reduced PGAP-induced MDA formation and [^{14}C]-5-HT release by the resuspended platelets without affecting irreversible aggregation; higher concentrations of mepacrine abolished all three responses. Chlorpromazine, a calmodulin antagonist, similarly inhibited PGAP-induced MDA formation and irreversible aggregation, and at 100 μM abolished monophasic aggregation.

3 The cyclo-oxygenase inhibitor indomethacin caused a concentration-dependent reduction of PGAP-induced MDA formation by resuspended human platelets without significantly inhibiting [^{14}C]-5-HT release or irreversible aggregation; concentrations ($>$ 1.75 μM) which inhibited MDA formation by more than 94% abolished [^{14}C]-5-HT release, and converted second phase irreversible aggregation to an extensive reversible response.

4 2-Methylthioadenosine 5′-phosphate (2 methylthio-AMP), an ADP antagonist, inhibited PGAP-induced MDA formation, [^{14}C]-5-HT release and second phase aggregation in the human platelet suspensions in a parallel, concentration-dependent manner; at 9.4 μM 2-methylthio-AMP, both MDA formation and [^{14}C]-5-HT release were abolished and monophasic, reversible aggregation remained.

5 Albumin was required for aggregation of washed human platelets to PGAP. Irreversible PGAP-induced aggregation of washed [^{14}C]-arachidonate-labelled platelets was accompanied by a low net loss of ^{14}C from platelet phospholipids, an equivalent increase in ^{14}C in free fatty acids, and the appearance of ^{14}C in thromboxane (Tx)B_2; mepacrine reduced the loss in ^{14}C from phospolipids and inhibited aggregation and formation of [^{14}C]-TxA_2. Thrombin-induced aggregation was accompanied by substantial loss of ^{14}C from phospholipids and equivalent gains of ^{14}C in free fatty acids and TxB_2; mepacrine pretreatment caused partial inhibition of thrombin-induced aggregation, halved the net ^{14}C loss from phospholipids, but had little effect on the appearance of ^{14}C in TxB_2.

6 It is concluded that in human platelets PGAP-induced dense granule release and irreversible aggregation are dependent on the liberation of arachidonate and its metabolism via prostaglandin endoperoxides to thromboxane, that PGAP and thrombin elicit mobilization of arachidonate from different pools of membrane phospholipids, and that the mechanism of PGAP-activation of human platelets differs from those of thrombin- and ADP-activation.

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Introduction

Palmitaldehyde, olealdehyde and linolealdehyde acetal phosphatidic acids, the major components of the smooth muscle-contracting phospholipid tissue extract Darmstoff (Vogt, 1949; Wiley *et al.*, 1970), induced a dose-dependent aggregation of human and sheep platelets in platelet-rich plasma (Brammer *et al.*, 1983). The most potent of the three acetal phosphatic acids, the palmitaldehyde congener (PGAP), had a threshold concentration of 2.5–5 μM for aggregation of human platelets in platelet-rich plasma. Irreversible second phase aggregation of human platelets by PGAP was associated with the release reaction, and release did not accompany the reversible first phase aggregation induced by lower concentrations of PGAP. Comparative studies of the inhibition of PGAP- and adenosine 5′-pyrophosphate (ADP)-induced human platelet aggregation and release by selected inhibitors indicated, albeit indirectly, that PGAP-induced second phase irreversible aggregation is mediated by cyclo-oxygenase metabolites of arachidonic acid, and that aggregation to PGAP involves promotion of calcium flux or intraplatelet calcium mobilization. We report here further studies of the nature of the platelet-stimulant actions of PGAP. The linkage of arachidonate metabolism, dense granule release and aggregation in PGAP stimulation has been investigated by monitoring malondialdehyde formation and 5-hydroxytryptamine (5-HT) release in aggregating platelets in the absence and presence of drugs which inhibit phospholipase A_2 and cyclo-oxygenase, and of an ADP antagonist. In addition, platelet aggregation to PGAP and thrombin and the concomitant metabolism of arachidonic acid released from platelet phospholipid have been compared using platelets labelled with [^{14}C]-arachidonic acid. Our findings demonstrate that de-esterification of arachidonate from membrane phospholipid, and its metabolism to thromboxane (Tx)A_2 are required for irreversible aggregation to PGAP, and that irreversible aggregation induced by PGAP is associated with substantially less hydrolysis of arachidonate from platelet phospholipid than occurs with aggregation induced by thrombin.

Methods

Resuspended human platelets

Blood, obtained by venipuncture from fasting volunteers who had not ingested any drugs for 10 days, was mixed with one-twelfth volume of acid-citrate dextrose anticoagulant (composition: 0.17 M trisodium citrate, 0.3 M citric acid, 4% dextrose) and centrifuged at 175 *g* for 20 min. Platelet-rich plasma (PRP) was aspirated, made 10 mM with regard to ethylenediaminetetracetic acid (EDTA) and centrifuged first at 1,360 *g* for 10 min and then at 1,650 *g* for 5 min. Platelet-free plasma was removed and the platelets were resuspended at a concentration of 1×10^9 platelets per ml in modified Tyrode solution (composition (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 12, NaH_2PO_4 0.36, $MgCl_2$ 1 and glucose 5.6, adjusted to pH 7.35) which contained 3.5 mg of bovine serum albumin and 0.5 units of apyrase-ADPase activity per ml. When ADP was to be used as the platelet stimulant, thrombin-free fibrinogen (Mustard *et al.*, 1975) was added to the platelet suspension to give a final concentration of 1 mg ml^{-1}.

[^{14}C]-arachidonate-labelled washed platelets

Sixty-four ml of PRP were incubated with 0.022 μmol (1.25 μCi) of [^{14}C]-arachidonic acid for 2 h at 37°C. The PRP was cooled to 4°C, made 10 mM with regard to EDTA and centrifuged at 1,360 *g* for 20 min then at 1,650 *g* for 5 min. The supernate containing unincorporated [^{14}C]-arachidonic acid was removed, and the platelet pellet was suspended in an equal volume of modified Tyrode solution (for composition see above) containing 3.5 mg of bovine serum albumin and 0.5 units of apyrase-ADPase activity per ml. The platelets were recentrifuged, the supernatant was aspirated and the platelets were resuspended in 18.6 ml of the same modified Tyrode solution containing albumin and apyrase.

Platelet aggregation

Platelet aggregation was monitored turbidimetrically at 37°C as described previously (Brammer *et al.*, 1983), using 0.5 ml samples of resuspended platelets (RSP) or 3.0 ml samples of [^{14}C]-arachidonate-labelled washed platelet suspension; platelet samples were stirred at 1,000 r.p.m. Aggregating agents and inhibitors were added to the RSP samples in volumes of 5 μl, and to samples of the [^{14}C]-arachidonate-labelled platelet suspension in volumes such that the total added volume was 100 μl. Control assays received equivalent volumes of 0.9% w/v NaCl solution (saline) or the appropriate vehicle. Inhibitors were preincubated in the samples for 1 min with stirring before addition of aggregating agents. Aggregation was quantitated by measuring the initial rate of increase in optical density (arbitrary units) and/or the maximum increase in optical density. For measurement of malondialdehyde (MDA) production by RSP samples, 30 μl of saline were added 4 min after the aggregating stimulus, the samples were im-

mediately acidified with 50 μl of 100% (w/v) trichloracetic acid in 3 M HCl and then analysed for MDA as described below. When the [^{14}C] distribution in lipids of [^{14}C]-arachidonate-labelled platelets was to be analysed, reaction was terminated 4 min after the aggregating stimulus by addition of 0.8 ml of 0.1 M EDTA.

Analysis of platelet malondialdehyde

Acidified platelet samples were mixed with 10 μl of 0.6% butylated hydroxytoluene in ethanol and centrifuged to yield clear supernates; 0.5 ml aliquots were withdrawn, mixed with 100 μl of 0.12 M thiobarbituric acid in 0.26 M Tris-HCl pH 7.0 and heated at 75°C for 30 min. Absorbance at 532 nm was measured and converted to nmol of MDA by comparison with an MDA standard curve; MDA was prepared by acid hydrolysis of malondialdehyde tetramethyl acetal. Subtraction of the MDA measured in control assays of unstimulated platelet samples gave the amount of MDA formed by stimulated platelets; this was expressed as nmol per 10^9 platelets. The lowest detectable amount of MDA per assay was approximately 10 pmol.

Release of platelet [^{14}C]-5-hydroxytryptamine

Ten ml of RSP were incubated with 1 μCi of [^{14}C]-5-HT at 37°C for 1 h. Aggregation was monitored in 0.5 ml samples of the labelled platelets as described above. Duplicate 200 μl aliquots of the sample were taken 4 min after addition of the aggregating agent for measurement of [^{14}C]-5-HT release, as described earlier (Brammer *et al.*, 1983). Release was expressed as a percentage of the total [^{14}C]-5-HT uptake, which was determined as the difference between the plasma ^{14}C level of the platelet suspension at the beginning and at the end of the incubation.

Analysis of the distribution of ^{14}C in lipids of [^{14}C]-arachidonate-labelled platelets

Lipids were extracted from quenched [^{14}C]-arachidonate-labelled platelet samples and the extracts were fractionated essentially as described by Bills *et al.* (1976). Each lipid extract was separated by column chromatography on activated silica acid into three fractions: A, neutral lipid + free fatty acids; B, prostaglandin + thromboxane; C, phospholipids. The fractions were then further resolved by thin layer chromatrography (t.l.c.). Chromatograms of fraction A (developed on silver-impregnated plates) were scraped from the origin to the solvent front in increments of 0.5 cm and the ^{14}C-activity of each 0.5 cm segment was measured. Graphs of d.p.m. versus migration distance were constructed. Chromatograms of [^{14}C]-arachidonic acid standards were processed identically. The ^{14}C-activity of each peak was obtained from the fractional weight of the peak and the total ^{14}C-activity of fraction A. Thin layer chromatograms of fraction B were developed with a reference standard of thromboxane B_2 and chromatograms of fraction C were developed using phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and phosphatidylserine as reference standards; these chromatograms were visualized by exposure to iodine vapour. Iodine-absorbing spots and the remainder of each migration path were scraped for ^{14}C measurement.

The ^{14}C content of organic samples (20–100 μl) and scrapings from thin layer chromatograms were vortexed with 10 ml of scintillation fluid containing 5 g of POP and 0.5 g of POPOP per 1 of toluene. Aqueous samples (0.1 ml) were solubilized with 0.4 ml of NCS tissue solubilizer; 9.5 ml of the scintillation fluid was added. ^{14}C was counted in a Packard Tricarb 3385 liquid scintillation spectrometer and counts were converted to d.p.m. by quench correction using external standard channels ratio.

Platelet counts

Platelet counts were performed by the Breker-Cronkite method using phase contract microscopy.

Statistics

Where results are given as means, ± s.e.mean is indicated. In figures standard errors are shown by vertical bars (except where the standard error is contained within the symbol). The significance of the difference between two means was calculated using Student's *t* test.

Materials

Monosodium palmitaldehyde acetal phosphatidic acid was supplied by Dr R.A. Wiley, University of Kansas. The synthesis of 2-methylthio-AMP has been previously described (Gough *et al.*, 1978). Thrombin (grade II from bovine plasma), albumin (bovine fraction V), 2,6-di-*t*-butyl-*p*-cresol, mepacrine (quinacrine dihydrochloride), chlorpromazine, indomethacin, arachidonic acid and apyrase (1.2–3.4 units of ADPase mg^{-1} protein) were obtained from the Sigma Chemical Company. Thromboxane B_2 was a gift from Dr John Pike from the Upjohn Company. Phospholipids were obtained from Supelco.

2[^{14}C]-5-hydroxytryptamine binoxalate (specific activity 51.5 mCi $mmol^{-1}$) was obtained from New England Nuclear. [^{14}C]-arachidonic acid (specific activity 58 mCi $mmol^{-1}$) and NCS tissue solubilizer

were supplied by Amersham. Lyophilized human fibrinogen, Grade L, was obtained from the Kabi Group Inc., Sweden. All other chemicals and reagents were the best grade commercially available. Unisil acid-washed 200–235 mesh activated silicic acid was obtained from Clarkson Chemical Company Incorporated. 'Uniplates' 250 μm silica gel G with 20% silver nitrate were supplied by Analtech, 250 μm silica gel G without gypsum Polygram plates by Brinkman Instruments and 250 μm silica gel 60 F_{254} aluminum plates by E. Merk, Darmstadt, Germany.

Results

Malondialdehyde formation, release of [^{14}C]-5-hydroxytryptamine and aggregation in resuspended human platelets stimulated by PGAP and ADP

Resuspended human platelets stimulated with PGAP formed MDA in an all-or-none fashion. Concentrations of PGAP which elicited only reversible aggregation did not stimulate MDA formation, while all concentrations of PGAP which induced biphasic irreversible aggregation caused MDA production (Figure 1); the mean yield of MDA for all these concentrations of PGAP was 1.02 ± 0.029 nmol per 10^9 platelets ($n = 5$). ADP similarly stimulated MDA

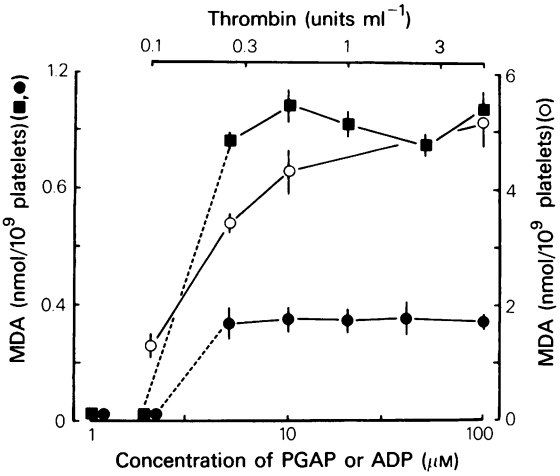

Figure 1 Log concentration-effect curves for human platelet MDA formation induced by PGAP, ADP and thombin. Five hundred μl samples of human platelets resuspended at 1×10^9 platelets ml^{-1} in a modified Tyrode solution containing 3.5 mg of bovine serum albumin and 0.5 units of apyrase ADPase activity per ml, (RSP), were stirred at 1,000 r.p.m. and 37°C in the aggregometer. Four min after initiation of aggregation with PGAP (■), ADP (●) or thrombin (○) the reaction was quenched and MDA was measured as described under Methods. Data points are means ± s.e.mean (vertical bars) of triplicate assays. Thrombin concentration is expressed as units per ml of platelet suspension.

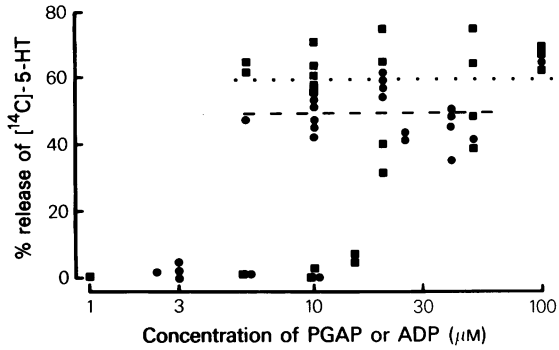

Figure 2 Concentration-response relationship of release of [^{14}C]-5-hydroxytryptamine ([^{14}C]-5-HT) from resuspended human platelets (RSP) stimulated with PGAP and ADP. Release was measured in 500 μl samples of [^{14}C]-5-HT-labelled RSP (see legend to Figure 1) 4 min after initiation of aggregation with PGAP (■) or ADP (●) as described under Methods, and is expressed as a % of initial platelet ^{14}C content. Each symbol represents the mean of duplicate measurements of one assay. Lines indicate the level of mean percentage release for all concentrations of PGAP and ADP associated with second phase aggregation: PGAP, 58.7 ± 2.8% ($n = 18$); ADP, 49.1 ± 1.9, ($n = 17$).

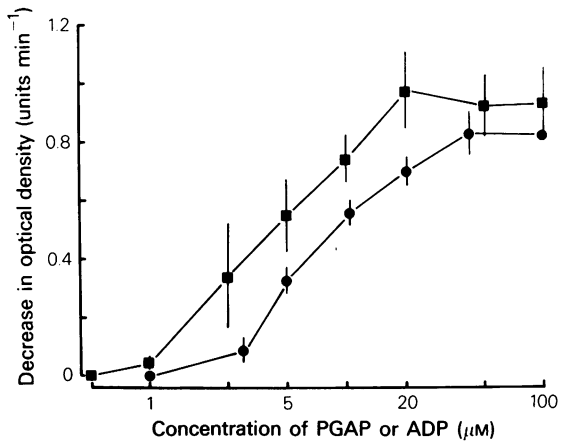

Figure 3 Log concentration-effect curves for the initial rate of aggregation of resuspended human platelets (RSP) stimulated with PGAP and ADP. Five hundred μl samples of RSP (see legend to Figure 1) were stirred at 1,000 r.p.m. at 37°C in the aggregometer and the optical density (arbitrary units) was recorded after the addition of PGAP (■) or ADP (●). Symbols represent means ± s.e.mean (vertical bars) of 4–26 assays (PGAP) or 3–23 assays (ADP).

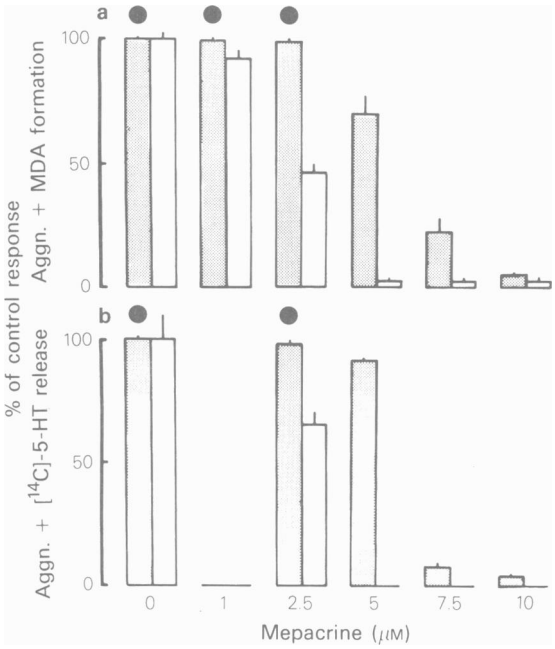

Figure 4 Comparison of the effects of mepacrine on PGAP-induced MDA formation, [^{14}C]-5-hydroxytryptamine release and aggregation (aggn.) in resuspended human platelets (RSP). Five hundred μl samples of RSP (see legend to Figure 1) either unlabelled or labelled with [^{14}C]-5-HT were stirred in the aggregometer at 1,000 r.p.m. and 37°C without or with mepacrine at the concentrations indicated. After 1 min PGAP was added to give final concentration of 20 μM. Aggregation was monitored for 4 min and samples were quenched with acid and assayed for MDA, or treated with EDTA and assayed for [^{14}C]-5-HT release, as described under Methods. Stippled columns indicate % of control maximum aggregation. Symbol ● shows that aggregation was irreversible. Open columns indicate % of control MDA formation (a) or of control [^{14}C]-5-HT release (b). Column heights give means ± s.e.mean (vertical bars) of triplicate assays.

formation in RSP in an all-or-none fashion, but the yield of MDA, 0.344 ± 0.004 nmol per 10^9 platelets ($n = 5$), was less than half that induced by PGAP (Figure 1). In contrast, as shown in Figure 1, thrombin caused dose-dependent formation of MDA, with a maximum yield of 5.14 ± 0.429 nmol of MDA per 10^9 platelets at 5 units of thrombin per ml of RSP. The release of [^{14}C]-5-HT from prelabelled RSP elicited by both PGAP and ADP was also all-or-none (Figure 2); PGAP was more effective, inducing release of 58.7 ± 2.8% ($n = 18$) of the platelet [^{14}C]-5-HT, compared to 49.1 ± 1% ($n = 17$) released by ADP, a difference of 9.6% ($P < 0.01$).

As was observed with human platelets in PRP (Brammer *et al.*, 1983), PGAP induced a rapid shape change in RSP followed by dose-dependent aggregation at higher concentrations of PGAP. The log concentration-effect curve for the initial rate of PGAP-induced aggregation was essentially parallel to that for ADP-induced aggregation (Figure 3). PGAP had a threshold concentration for aggregation of 0.5–1 μM and an EC_{50} of 4 μM, and was twice as potent as ADP, which had an EC_{50} of 8 μM. The maximum rate of aggregation was observed at > 20 μM PGAP.

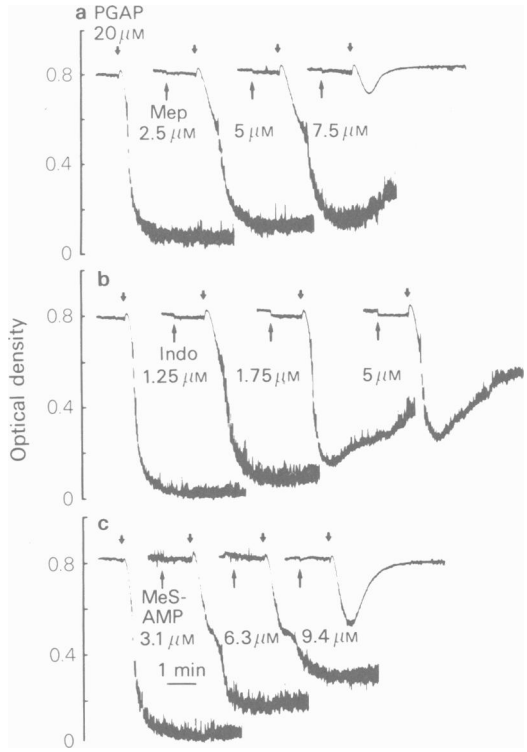

Figure 5 Effects of mepacrine, indomethacin and 2-methylthio-AMP on PGAP-induced aggregation in samples of resuspended human platelets (RSP). Five hundred μl samples of RSP (see legend to Figure 1) either unlabelled or labelled with [^{14}C]-5-hydroxytryptamine were stirred in the aggregometer with the indicated concentrations of (a) mepacrine (Mep), (b) indomethacin (Indo) or (c) methylthio-AMP (MeS-AMP) for 1 min before stimulation with PGAP (final concentration, 20 μM), shown by the upper arrows in each panel. Optical density (arbitrary units) was recorded for 4 min. Each tracing is representative of 6 assays in 2 preparations of RSP.

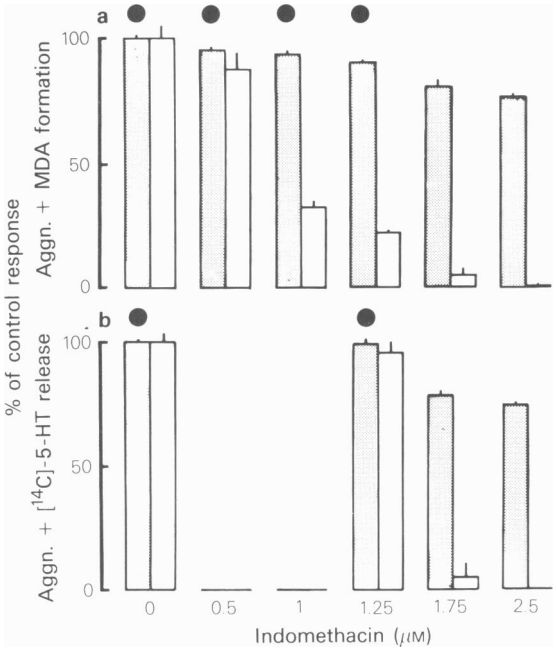

Figure 6 Comparison of the effects of indomethacin on (a) PGAP-induced MDA formation, (b) [^{14}C]-5-hydroxytryptamine (5-HT) release and aggregation (aggn.) in resuspended human platelets (RSP). See legend to Figure 4 for explanation of methods. Stippled columns indicate % of control maximum aggregation. Symbol ● shows that aggregation was irreversible. Open columns indicate % of control MDA formation (a) or of control [^{14}C]-5-HT release (b). Column heights give means ± s.e.mean (vertical bars) of triplicate assays.

Effects of drugs on malondialdehyde formation, [^{14}C]-5-hydroxyptamine (5-HT) release and aggregation in PGAP-stimulated human platelets

Mepacrine Mepacrine, a platelet phospholipase A_2 inhibitor (Blackwell, *et al.*, 1977), caused dose-dependent inhibition of MDA formation in PGAP-stimulated platelets (Figure 4). At 2.5 μM mepacrine reduced both MDA formation and [^{14}C]-5-HT release to 46.4 ± 3.4% and 65.9 ± 4.3%, respectively, of the control values, without affecting irreversible aggregation of the RSP (Figures 4, 5). Five μM mepacrine abolished PGAP-induced MDA formation and [^{14}C]-5-HT release and concomitantly converted aggregation to a reversible biphasic response which reached 70–90% of control maximum aggregation (Figures 4, 5); 10 μM mepacrine virtually completely antagonized aggregation to PGAP.

Indomethacin Malondialdehyde formation in PGAP-stimulated platelets was inhibited dose-dependently by the cyclo-oxygenase inhibitor, indomethacin (Figure 6); [^{14}C]-5-HT release and aggregation were less sensitive than MDA formation to antagonism by indomethacin. When MDA formation was reduced to 22.1 ± 0.8% of control by 1.25 μM indomethacin, no reduction of [^{14}C]-5-HT release was observed and only a small reduction (to 91.7 ± 0.5% of control) in the extent of irreversible aggregation occurred (Figures 5 and 6). When the indomethacin concentration was increased to 1.75 μM, both MDA formation and [^{14}C]-5-HT release were reduced to less than 6% of control values, and aggregation was altered to an extensive reversible response, with a maximum which was 80% of control. Higher concentrations of indomethacin abolished MDA formation and [^{14}C]-5-HT release, but the extent of reversible aggregation was not reduced below 73% of the aggregation maximum observed in control responses (Figures 5 and 6).

2-Methylthio-AMP Malondialdehyde formation, [^{14}C]-5-HT release and second phase aggregation in

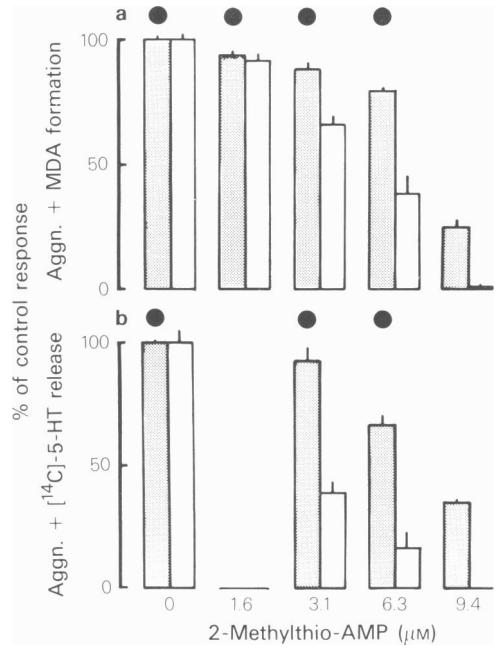

Figure 7 Comparison of the effects of 2-methylthio-AMP on (a) PGAP-induced MDA formation, (b) [^{14}C]-5-hydroxytryptamine (5-HT) release and aggregation (aggn.) in resuspended human platelets (RSP). See legend to Figure 4 for explanation of methods. Stippled columns indicate % of control maximum aggregation. Symbol ● shows that aggregation was irreversible. Open columns indicate % of control MDA formation (a) or of control [^{14}C]-5-HT release (b). Column heights give means ± s.e.mean (vertical bars) of triplicate assays.

PGAP-stimulated RSP were all inhibited in a concentration-dependent manner by 2-methylthio-AMP 1.6–9.4 μM (Figures 5 and 7). This inhibitor, which is a specific antagonist of ADP-induced platelet aggregation (Gough *et al.*, 1978; Maguire, 1981), sharply reduced the maximum change in optical density of those aggregation curves which exhibited a second phase, without causing disaggregation, in contrast to the observations obtained with indomethacin and mepacrine (Figure 5). At 9.4 μM, 2-methylthio-AMP abolished second phase aggregation, [^{14}C]-5-HT release and MDA formation.

Chlorpromazine Malondialdehyde formation in PGAP-stimulated RSP was inhibited in a dose-dependent fashion by chlorpromazine, a calmodulin antagonist (Triggle, 1982); at 37.5 μM chlorpromazine, MDA formation by PGAP-stimulated platelets was abolished (Figure 8). Irreversible aggregation was not affected by concentrations of chlorpromazine up to 31.2 μM even though MDA formation was inhibited by more than 70%. However, as shown in Figure 8, when MDA formation was completely blocked by >37.5 μM chlorpromazine, diminished reversible aggregation was observed; this response was abolished by 100 μM chlorpromazine (data not shown).

PGAP-induced aggregation of washed human platelets

Platelets which were isolated from PRP and washed, as described under Methods for the preparation of

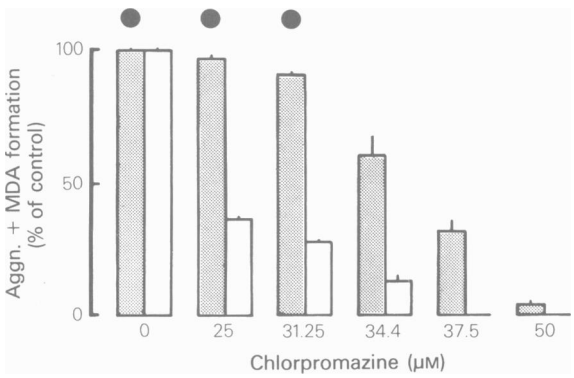

Figure 8 Comparison of the effects of chlorpromazine on PGAP-induced MDA formation and aggregation (aggn.) in resuspended human platelets (RSP). See legend to Figure 4 for explanation of methods. Stippled columns indicate % of control maximum aggregation. Symbol ● shows that aggregation was irreversible. Open columns indicate % of control MDA formation. Column heights give means ± s.e.mean (vertical bars) of triplicate assays.

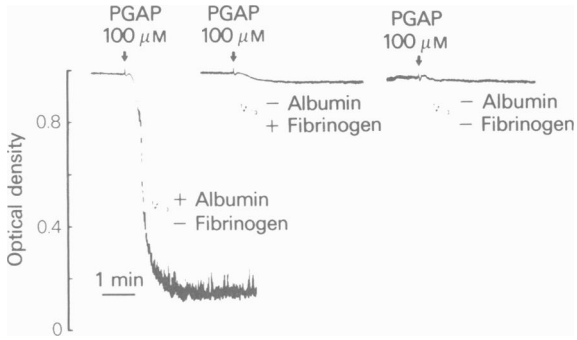

Figure 9 Albumin requirement for PGAP-induced aggregation of washed human platelets. Human platelets, washed as described under Methods for the preparation of [^{14}C]-arachidonate-labelled washed platelets were finally suspended in modified Tyrode solution without protein, or in the same solution containing either 0.35 g% of albumin or 0.1 g% of thrombin-free fibrinogen. PGAP was added at the arrow to stirred 500 μl platelet samples to give a final concentration of 100 μM, and optical density was recorded. Each tracing is typical of 3.

[^{14}C]-arachidonate-labelled washed platelets, were finally suspended in the modified Tyrode solution without protein, or in the same solution containing either 0.35 g% of albumin or 0.1 g% of thrombin-free fibrinogen. As shown in Figure 9, 100 μM PGAP did not cause aggregation in the absence of protein and induced only a minimal response in the presence of fibrinogen, but caused maximal aggregation when the washed platelets were suspended in modified Tyrode solution containing albumin.

Distribution of ^{14}C in lipids of control and stimulated [^{14}C]-arachidonate-labelled platelets

Human platelets incubated in PRP with [^{14}C]-arachidonate took up 37.6% of the label; after 2 washes the platelets retained 34.2% of the [^{14}C]-arachidonate. As shown in Figure 10, 5 units per ml of thrombin, 20 μM PGAP and 20 μM ADP elicited maximal aggregation of the washed labelled platelets; 20 μM mepacrine abolished the aggregation to PGAP and reduced the aggregation to thrombin.

Lipid extraction of the stimulated [^{14}C]-arachidonate-labelled platelet samples, and of a saline-treated control, followed by fractionation of the lipid extract and thin layer chromatographic analysis of the resulting fractions resulted in an average recovery of the total ^{14}C content of the samples of 96.0 ± 0.2% ($n=6$), i.e. 152,162 ± 1,454 d.p.m. ($n=6$) were extracted from the samples and recovered as free lipids and phospholipids. As shown in Table 1, free fatty acids (fraction A) accounted for

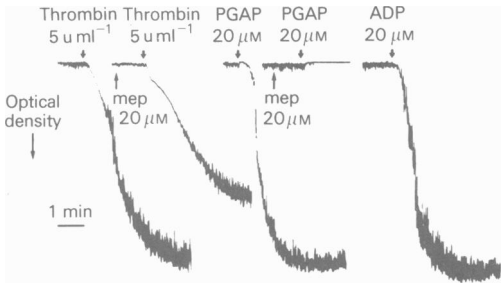

Figure 10 Aggregation of [^{14}C]-arachidonic acid-labelled washed platelets by thrombin and PGAP in the absence and presence of 20 μM mepacrine (mep), and by ADP. Either saline or mepacrine in saline was added to 3 ml samples of the stirred platelet suspension in 1.1 cm path-length aggregation cuvettes; 1 min later thrombin, PGAP or ADP were added as indicated by the arrows. Optical density was recorded for 4 min and samples were treated with 0.8 ml of 0.1 M EDTA. Final concentrations are indicated. The sample which received ADP was pre-treated with thrombin-free fibrinogen, final concentration 0.1 g%. Details are given under Methods.

4.2% of the total lipid ^{14}C extracted from saline-treated control platelets. Radiochromatographic analysis of fraction A yielded four well-resolved zones; the R_F0.7 zone was identified as [^{14}C]-arachidonic acid by comparison with a reference standard of [^{14}C]-arachidonic acid and contained 0.5% of the total recovered d.p.m. Zones of R_F0.85–0.9 and R_F0.35–0.55 were identified as 17-hydroxyheptatrienoic and 12-hydroxyeicosatetraenoic acids, respectively, by comparison with published R_F values (Bills *et al.*, 1976), but as reference standards were not available to confirm the assignments, the radioactivity contained in these zones was combined with that in a zone near the origin and termed 'other'. No [^{14}C]-thromboxane B_2 was detected in the saline-treated platelets and the remaining 95.8% of the incorporated ^{14}C was found in fraction C, the phospholipid fraction (Table 1). T.l.c. of reference standards showed that phosphatidylinositol and phosphatidylserine had identical R_F values, and that the combined phosphatidylinositol-phosphatidylserine spot, phosphatidylcholine and phosphatidylethanolamine were well resolved. Iodine-absorbing spots for which reference standards were not available were found on the origin and at R_F0.17 on chromatograms of fraction C, and were tentatively identified as lysophospholipid and sphingomyelin, respectively, by comparison with published R_F values (Bills *et al.*, 1976); the radioactivity found in these spots is described as 'other'. The distribution of ^{14}C in the platelet lipids was in excellent agreement with that found by Bills *et al.* (1976).

When the suspension of washed [^{14}C]-arachidonate-labelled platelets was irreversibly aggregated with 20 μM PGAP (Figure 10), the distribution of ^{14}C in platelet lipids was modified, and 0.3% of the total ^{14}C content appeared in thromboxane B_2 (Table 1). As shown in Table 1, a 1.3% net

Table 1 Distribution of ^{14}C in lipids of control (saline-treated) and PGAP- and thrombin-stimulated [^{14}C]-arachidonate-labelled human platelets

	*% of total d.p.m.**				
	Control (saline-treated)	*PGAP-stimulated*	*Mepacrine-treated, PGAP-stimulated*	*Thrombin-stimulated*	*Mepacrine-treated, thrombin-stimulated*
Lipid fraction					
A: Arachidonate	0.5	1.3	1.3	6.5	6.5
Other	3.7	4.1	3.9	17.1	8.0
Total A	4.2	5.4	4.9	23.6	14.5
B: Thromboxane B_2	—	0.3	<0.05	0.5	0.4
C: Phosphatidylcholine	56.9	52.8	57.0	39.6	49
Phosphatidylinositol + phosphatidylserine	25.2	24.3	23.1	19.1	20.8
Phosphatidyl-ethanolamine	10.3	12.7	11.6	11.4	17.3
Other	3.4	4.7	3.5	6.0	0.3
Total C	95.8	94.5	95.1	76.1	85.1

* Average total d.p.m. recovered in 6 separate analyses = 152,162 ± 1000. Total d.p.m. in fractions A, B and C in the saline-treated control platelets were A, 6387; B, 0; C, 145,775.

Final concentrations of PGAP, thrombin and mepacrine were 20 μM, 5 units ml^{-1} and 20 μM, respectively; for details see legend to Figure 10.

reduction of the ^{14}C located in phospholipids occurred, and there was a net increase of 1.2% in the ^{14}C content of the free fatty acid fraction; the latter increase was comprised largely of the increase in arachidonic acid ^{14}C. Mepacrine pretreatment completely blocked aggregation to 20 μM PGAP (Figure 10), essentially abolished the formation of [^{14}C]-thromboxane B_2, and reduced the PGAP-induced net release of ^{14}C from phospholipids (Table 1).

Maximal aggregation of the labelled platelet suspension to thrombin (Figure 10) was associated with the appearance of 0.5% of the total ^{14}C in the thromboxane B_2 fraction and a net gain of 19.4% of total ^{14}C in free fatty acids; of this latter gain, one third (or 6% of the total recovered ^{14}C) was due to an increase in [^{14}C]-arachidonic acid. A net loss of 19.9% ^{14}C from the phospholipid fraction occurred. Within the component phospholipids 15.3% and 6.1% losses in ^{14}C took place from phosphatidylcholine and phosphatidylinositol plus phosphatidylserine, respectively (Table 1). Mepacrine partially inhibited aggregation of the labelled platelets to thrombin (Figure 10). When compared with the distribution of ^{14}C in lipids of uninhibited thrombin-induced aggregates, the ^{14}C distribution in the mepacrine-inhibited aggregates showed similar formation of [^{14}C]-thromboxane, but less net accumulation of ^{14}C in the free fatty acid fraction (14.5% cf. to 23.6%), and reduced net loss of ^{14}C from the phospholipid fraction (10.7% cf. 19.7%); the formation of [^{14}C]-arachidonic acid was the same in both mepacrine-treated and mepacrine-untreated thrombin-aggregated platelets (Table 1).

Discussion

Palmitaldehyde acetal phosphatidic acid caused dose-dependent aggregation of human platelets resuspended in modified Tyrode medium, and at concentrations which caused irreversible aggregation, elicited [^{14}C]-5-HT release from prelabelled platelets. The resuspended platelets were more sensitive to activation by PGAP than were platelets in PRP (Brammer *et al.*, 1983), the EC_{50} values for aggregation being 4 μM and 8 μM, respectively. Moreover PGAP released 14.7% more [^{14}C]-5-HT from RSP than from platelets in PRP: $58.7 \pm 2.8\%$ compared to $44.0 \pm 2.4\%$ ($P < 0.001$). In RSP, PGAP was more potent than ADP with regard to aggregation and caused 9.6% more release of [^{14}C]-5-HT, while in PRP, PGAP was less potent than ADP, and elicited 14% ($P < 0.001$) less release of platelet [^{14}C]-5-HT (Brammer *et al.*, 1983). The greater potency of PGAP for RSP as compared to platelets in PRP could be due to binding of PGAP in PRP by plasma protein or lipoprotein, resulting in a reduction in its effective concentration. Responses to PGAP in PRP which contained visible lipid, presumably chylomicrons, were reduced in comparison with responses obtained in chylomicron-free PRP (Brammer & Maguire, unpublished observations). Alternatively the process of resuspension may have sensitized the platelets to PGAP, perhaps unmasking membrane receptors. It is likely that apyrase in the resuspending Tyrode solution contributed to the reduced potency of ADP in RSP compared to PRP.

The formation of MDA by PGAP- and ADP-stimulated platelets was not dose-dependent, but was observed only at those agonist concentrations which elicited irreversible aggregation; PGAP was the more potent, producing three times more MDA than was produced by ADP. Increasing the concentration of either agent above the threshold for MDA production did not result in additional formation of MDA. Thus, in platelets stimulated by PGAP and ADP, irreversible aggregation is linked with both [^{14}C]-5-HT release, a measure of dense granule release, and with MDA formation, an indicator of arachidonate liberation and metabolism via cyclo-oxygenase and thromboxane synthetase (Moncada & Vane, 1978).

Mepacrine, a drug which inhibits arachidonate mobilization in collagen- and thrombin-stimulated platelets (Blackwell *et al.*, 1977; Hofmann *et al.*, 1982), was a potent dose-dependent inhibitor of MDA formation in PGAP-stimulated platelets. A mepacrine-induced reduction in MDA yield of 55% was associated with a 30% reduction in [^{14}C]-5-HT release, but with only minimal inhibition of the irreversible aggregation. However, a higher concentration of mepacrine, which abolished MDA formation, concomitantly abolished release and converted irreversible aggregation to an extensive reversible response. These findings suggest that PGAP-induced dense granule release and irreversible aggregation are dependent on the mobilization of arachidonate from platelet phospholipids, and its metabolism to thromboxane. The actions of chlorpromazine on MDA formation and aggregation were similar to those of mepacrine; at higher concentrations both drugs completely inhibited primary aggregation. Chlorpromazine, a calmodulin antagonist, could indirectly inhibit calcium-stimulated phospholipases and has been shown to inhibit arachidonate mobilization in thrombin-stimulated platelets (Walenga *et al.*, 1981). Mepacrine may inhibit arachidonate release directly via inhibition of phospholipase A_2 (Blackwell *et al.*, 1977) or phosphatidylinositol-specific phospholipase C (Hofman *et al.*, 1982), or could act indirectly by antagonizing calmodulin (Volpi *et al.*, 1981). Furthermore chlorpromazine, a phenothiazine neuroleptic, and mepacrine, an amphiphilic cation, have local anaesthetic, 'membrane-

stabilising' actions, which may include inhibition of Ca^{2+} fluxes (Seeman, 1972); the former has been shown to inhibit transmembrane Ca^{2+} influx in rabbit aortic strips (Kanamori *et al.*, 1981). These non-specific actions may contribute to the effects of the drugs on platelet arachidonate metabolism; as they both abolished the reversible first phase of aggregation this suggests the possibility of a role for Ca^{2+} flux in mediating this aspect of PGAP-induced platelet activation.

Indomethacin, a specific cyclo-oxygenase inhibitor, also caused dose-dependent inhibition of MDA formation in PGAP-activated platelets. Release of [^{14}C]-5-HT and the irreversible second phase of aggregation were abolished when the concentration of indomethacin was such as to reduce MDA formation by 94% or more, suggesting that dense granule release and irreversible second phase aggregation in PGAP-stimulated platelets are absolutely dependent on the formation of prostaglandin endoperoxides and thromboxane. Nevertheless, substantial inhibition of cyclo-oxygenase activity, indicated by a 78% reduction in MDA formation, occurred without commensurate reduction in [^{14}C]-5-HT release and irreversible aggregation; the latter responses remained at, or near, their control values. These findings suggest that, while synthesis of prostaglandin endoperoxides and thromboxane is required for dense granule release and irreversible aggregation in PGAP-activated platelets, optimal cyclo-oxygenase activity indicated by optimal MDA formation is not essential, and that dense granule release can be achieved when thromboxane synthesis is sub-optimal. Similar findings were obtained in studies with vinblastine, a drug which inhibits the thromboxane pathway of platelet arachidonate metabolism; vinblastine inhibited MDA formation in PGAP-stimulated RSP by 54% without reducing [^{14}C]-5-HT release and only minimally inhibiting irreversible aggregation (Brammer *et al.*, 1982). That thromboxane synthesis is not required for first phase aggregation to PGAP, is indicated by the extensive reversible aggregation which occurred in the presence of complete MDA blockade by indomethacin, and confirms similar conclusions reached from studies in PRP (Brammer *et al.*, 1983).

The ADP antagonist, 2-methylthio-AMP, inhibited all three platelet responses, MDA formation, dense granule release, and second phase irreversible aggregation, in concert in a concentration-dependent fashion, and in contrast with results obtained using indomethacin and mepacrine, dense granule release was inhibited more than MDA formation. These findings may be explained by hypothesizing that the action of released dense granule ADP at its extracellular receptor site establishes a mechanism through which arachidonic acid liberation, dense granule release and irreversible aggregation are controlled or synchronized.

In contrast to the all-or-none relationship between PGAP-mediated platelet MDA formation and PGAP concentration, the extent of thrombin-induced MDA formation depended on the thrombin dose. Furthermore, the maximum amount of MDA formed by thrombin-activated platelets was five times that formed by PGAP-activated platelets. Platelet MDA is formed from PGH_2 via the action of thromboxane synthetase (Hamberg & Samuelsson, 1974) and its measurement serves as an indicator of both cyclo-oxygenase activity and of thromboxane A_2 formation (Diczfalusy *et al.*, 1977; McMillan *et al.*, 1978). Differences between the MDA yields obtained from PGAP- and thrombin-stimulated platelets may thus indicate differences in thromboxane A_2 formation and, as availability of arachidonate is the rate-limiting step in the pathway to MDA and thromboxane A_2, differences in degree and mode of arachidonate release. Thrombin caused almost 20% loss of ^{14}C from the phospholipid fraction of [^{14}C]-arachidonate-labelled platelets, 2% of which appeared as thromboxane B_2, the stable thromboxane A_2 metabolite, and 27% as unchanged [^{14}C]-arachidonic acid. The accumulation of unoxidized [^{14}C]-arachidonic acid and the small conversion of the released acid to [^{14}C]-thromboxane B_2 can be accounted for by binding of arachidonic acid by albumin in the suspension medium (Bills *et al.*, 1976; 1977; Stuart *et al.*, 1980). Mepacrine reduced the loss of ^{14}C from phospholipids by 9% without substantially modifying [^{14}C]-thromboxane formation, and concomitantly reduced aggregation. Irreversible aggregation of [^{14}C]-arachidonate-labelled platelets by PGAP was associated with a small loss of ^{14}C from the phospholipid pool, equal to one-fifteenth that elicited by thrombin, and with formation of [^{14}C]-thromboxane B_2; both aggregation and [^{14}C]-thromboxane formation were inhibited by mepacrine. Irreversible aggregation induced by ADP was associated with a similar small loss of ^{14}C from phospholipids (data not shown). Comparison of the ratio of the net ^{14}C liberated by thrombin, PGAP and ADP, 15:1:1, with the ratio of MDA yield achieved by the three agents, 15:3:1, indicates a correspondence between ^{14}C liberation and MDA formation for thrombin and ADP, but not for PGAP. This disparity suggests that the [^{14}C]-arachidonate released by PGAP has a lower specific activity than that released by thrombin (and ADP), and, *ipso facto*, that different pools of incorporated [^{14}C]-arachidonic acid participate in the liberation of arachidonate when platelets are activated by these agents. Activation by PGAP may result in release of arachidonate from a poorly labelled phospholipid pool and/or activation by thrombin may release arachidonate

from a pool which is enriched with [^{14}C]-arachidonate relative to the endogenous acid. [^{14}C]-arachidonic acid has been shown to be incorporated to different extents into the major platelet membrane phospholipids by Lagarde *et al.* (1982).

Platelet aggregation initiated by agents such as ADP, thrombin, adrenaline and platelet-activating factor is dependent on external fibrinogen and exposure of membrane fibrinogen receptors (Hawiger, *et al.*, 1980; Peerschke, 1982; Kloprogge *et al.*, 1983). As PGAP induces platelet aggregation in the absence of fibrinogen, PGAP may initiate aggregation by a unique mechanism. That albumin is required for PGAP-induced aggregation is also unique. The presence of albumin in platelet-suspending media serves to protect platelets, maintaining them in the disc shape, but is not critical for aggregation to a variety of platelet-activating agents (Kinlough-Rathbone *et al.*, 1977). Thus the permissive effect of albumin on PGAP-induced aggregation of washed platelets is unusual and further studies will be necessary to determine its role.

The findings recounted here show that dense granule release and irreversible aggregation in PGAP-activated human platelets, are dependent on the mobilization of arachidonate from membrane phospholipid and its conversion to thromboxane, and that thromboxane synthesis is not required for reversible first phase aggregation. Furthermore the extensive PGAP-induced reversible aggregation observed in the presence of cyclo-oxygenase blockade suggests the possibility that lipid metabolites formed as a concomitant of arachidonate mobilization may serve to amplify the initial action of PGAP at the platelet membrane; such lipid mediators could be e.g. 1,2-diacylglycerol, an activator of platelet protein kinase C, (Sano *et al.*, 1983), or phosphatidic and lysophosphatidic acids, postulated to be calcium ionophores (Gerrard *et al.*, 1981). That calcium flux is important in mediating first phase aggregation in PGAP-stimulated platelets is suggested by the complete inhibition of this response by the 'membrane-stabilizing' drugs, and is in agreement with similar findings in PRP (Brammer *et al.*, 1983). These drugs did not inhibit the first phase response to ADP in PRP, signifying that the mechanisms of activation of human platelets by PGAP and ADP are different (Brammer *et al.*, 1983), a conclusion which is substantiated here by demonstration of the differences in their effects on platelet arachidonate metabolism. The mechanism of platelet activation by PGAP is also shown to differ from that of thrombin. The novel acetal function of PGAP contributes strongly to its platelet-activating potency (Brammer *et al.*, 1983). It is proposed that stimulation of human platelets by PGAP involves action at a specific platelet receptor followed by initiation of calcium flux and of a lipolytic response; further studies will be required to establish temporal and other relationships between these receptor-linked events.

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