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Acetal phosphatidic acids: novel platelet aggregating agents

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1 Palmitaldehyde, olealdehyde and linolealdehyde acetal phosphatidic acids induced rapid shape change and dose-dependent biphasic aggregation of human platelets in platelet-rich plasma; aggregation was reversible at low doses and irreversible at high doses of the acetal phosphatidic acids. The palmitaldehyde congener elicited monophasic dose-dependent aggregation of sheep platelets in platelet-rich plasma.

2 The threshold concentration for palmitaldehyde acetal phosphatidic acid (PGAP)-induced platelet aggregation was 2.5–5 μM for human platelets and 0.25–0.5 μM for sheep platelets. PGAP was 4–5 times as potent versus human platelets as the olealdehyde and linolealdehyde acetal phosphatidic acids, which were equipotent.

3 PGAP-induced irreversible aggregation of [^{14}C]-5-hydroxytryptamine ([^{14}C]-5-HT)-labelled human platelets in platelet-rich plasma was accompanied by release of 44.0 ± 2.4% (s.e.) of the platelet [^{14}C]-5-HT; reversible aggregation was not associated with release. In contrast, PGAP-induced release of [^{14}C]-5-HT-labelled sheep platelets was dose-dependent.

4 The adenosine diphosphate (ADP) antagonist, 2-methylthio-AMP, and the cyclo-oxygenase inhibitor, aspirin, abolished PGAP-induced second phase aggregation and release in human platelets but did not affect the first, reversible, phase of aggregation. Both the first and second phases of PGAP-induced aggregation were abolished by chlorpromazine, by the phospholipase A_2 inhibitor, mepacrine, and by nmolar concentrations of prostaglandin E_1 (PGE_1); these agents abolished the second, but not the first phase of ADP-induced aggregation.

5 The related phospholipids, lecithin, lysolecithin and phosphatidic acid, at $< 100\,\mu$M, neither induced aggregation of human platelets in platelet-rich plasma, nor modified PGAP-induced aggregation; 1-palmityl lysophosphatidic acid elicited aggregation of human platelets at a threshold concentration of 100 μM.

6 It is concluded that the acetal phosphatidic acids induce platelet aggregation *per se* by direct action at the platelet membrane, and that the acetal function is of primary importance in their potent platelet-stimulating activity. Moreover, as the acetal phosphatidic acids are the major components of the smooth muscle-contracting acidic phospholipid tissue extract 'Darmstoff' (Vogt, 1949), their potent platelet-aggregating properties may be of physiological or pathological significance.

Introduction

An acidic phospholipid material which Vogt (1957) isolated by hot alkali treatment of horse intestine contracted *in vitro* preparations of visceral smooth muscle similarly to an unknown substance which he had observed in the bath fluid of isolated frog intestine and which he called 'Darmstoff' (Vogt, 1949; Kuck & Vogt, 1950). A phospholipid substance extracted from rabbit kidney under less rigorous conditions also contracted visceral smooth muscle (Dyer & Walaszek, 1968). The major components of both the horse intestine and rabbit kidney phospholipid extracts were shown by Wiley, Summer & Walaszek (1970) to be phosphorylated glyceryl acetals of palmitaldehyde, olealdehyde and linolealdehyde; the structures were confirmed by synthesis, and the olealdehyde derivative was shown to elicit contractions of the guinea-pig ileum identical to those of the natural phospholipid mixture. The linolealdehyde

derivative was less active, and the palmitaldehyde congener was inactive. The effects of two of the acetal phosphatidic acids on smooth muscle suggested that they may also have specific actions on platelets. We show here that all three acetal phosphatidic acids potently stimulate human and sheep platelets, causing aggregation and release of dense granule contents, and we consider the effects of selected inhibitory agents on the acetal phosphatidic acid-induced platelet response.

Preliminary accounts of some of these results have been given (Brammer, Walaszek & Maguire, 1980).

Methods

Platelet aggregation

Blood was taken by venipuncture from human volunteers who had taken no drugs for 10 days and mixed with sufficient 7.6% trisodium citrate anticoagulant to give a final citrate concentration of 0.38%. Sheep were bled from the jugular vein into sufficient 7.6% trisodium citrate to give 0.38% citrate concentration in blood. Platelet-rich plasma (PRP) was prepared from anticoagulated blood by centrifugation at 175 *g* for 20 min in the case of human blood, and for 25 min in the case of sheep blood; PRP was aspirated and stored at room temperature. Platelet aggregation was monitored turbidimetrically at 37°C in a Payton aggregometer coupled with a Varicord 43 recorder using 0.5 ml samples of PRP stirred at 1000 rev/min. Aggregating agents and inhibitors were added to platelet samples in volumes of 5 μl; control assays received 5 μl of the appropriate solvent. Inhibitors were preincubated in the assay for 1 min before the addition of the aggregating agent. Platelet aggregation was quantitated by measuring the initial rate of decrease in optical density (arbitrary units). Platelet counts were performed by the Brecker-Cronkite method using phase contrast microscopy and were typically from 4 to 6×10^8/ml for human PRP and from 7 to 10×10^8/ml for sheep PRP.

Platelet release reaction

Platelet-rich plasma was incubated with 0.1 μCi/ml of [^{14}C]-5-HT for 1 h at 37°C. Aggregation was monitored in 0.5 ml samples of the labelled platelets as described above. Four min after addition of the aggregating agent, duplicate 200 μl aliquots of the sample were taken for measurement of [^{14}C]-5-HT release, added to 20 μl of 77 mM EDTA, mixed and centrifuged at 10,000 *g* for 2 min. One hundred μl of each resulting supernate was digested with 0.8 ml of NCS tissue solubilizer in scintillation vials; 9.1 ml of toluene containing 5 g/l of PPO and 0.5 g/l of POPOP were added and ^{14}C was counted in a Packard 3385 scintillation spectrophotometer. Release was expressed as a percentage of the total platelet [^{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 1 h incubation period.

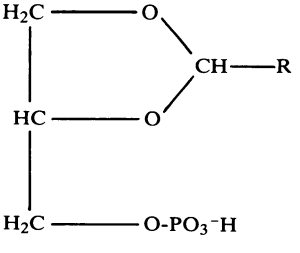
Presentation of observations

Where results of measurements of aggregation and release 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).

Materials

The monosodium salts of palmitaldehyde acetal phosphatidic acid (PGAP) and olealdehyde acetal phosphatidic acid (OGAP), and the disodium salt of linolealdehyde acetal phosphatidic acid (LGAP) (*cf.* Table 1), were synthesized as described (Wiley *et al.*, 1970). The samples were chromatographically homogeneous and had satisfactory elemental analyses. Lecithin (egg), phosphatidic acid (egg) and lysolecithin (egg) were obtained from Supelco, Inc. 1-Palmityl lysophosphatidic acid was supplied by Serdary Research Laboratories. The synthesis of 2-methylthio-AMP has been reported (Gough, Nobbs, Middleton, Penglis-Caredes & Maguire, 1978). Disodium adenosine 5′-diphosphate, theophylline, reserpine, mepacrine (quinacrine hydrochloride), and chlorpromazine were obtained from the Sigma Chemical Company, and aspirin from Aldrich Chemical Company. All other reagents were the highest grade commercially available. 2[^{14}C]-5-hydroxytryptamine binoxalate (51.5 mCi/mmol) was supplied by New England Nuclear and NCS tissue solubilizer by Amersham.

Table 1 Structure of acetal phosphatidic acids



R	*Abbreviation*
$-(CH_2)_{14}CH_3$	PGAP
$-(CH_2)_7CH=CH(CH_2)_7CH_3$	OGAP
$-(CH_2)_7CH=CHCH_2CH=CH(CH_2)_4CH_3$	LGAP

Preparation of drug solutions

Drug solutions were prepared fresh before each experiment. Phosphatidic acid and lysophosphatidic acid were dissolved in 0.9% w/v NaCl solution (saline) which was 10 mM with regard to NaOH. Lecithin and lysolecithin were dissolved in 50% aqueous ethanol (v:v), and aspirin was dissolved in 5% $NaHCO_3$. Solutions were diluted in saline for addition to platelet assays. Controls of the diluted solubilizing media had no direct effect on the platelets and no effect on responses induced by PGAP or ADP. Solutions of ADP, PGAP, OGAP, LGAP, mepacrine, and 2-methylthio-AMP were prepared in saline.

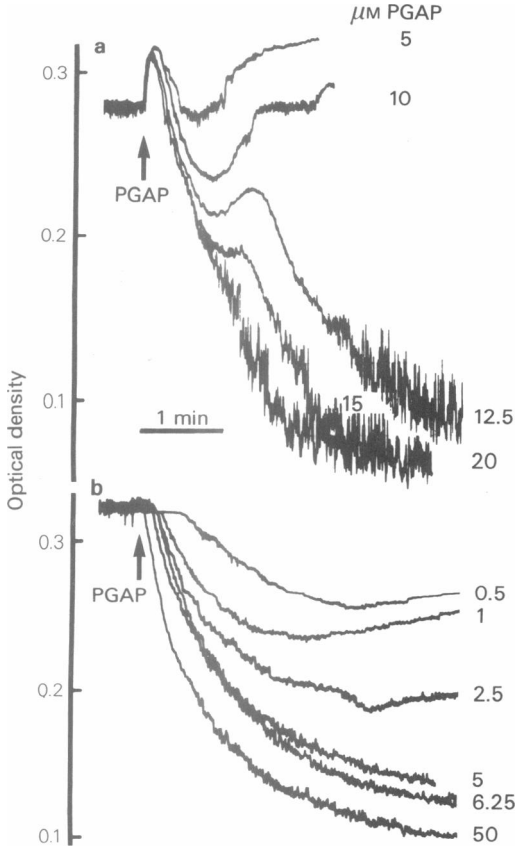


Figure 1 Palmitaldehyde acetal phosphatidic acid (PGAP)-induced aggregation of platelets in PRP. Samples of PRP were stirred at 1000 rev/min at 37°C in the aggregometer; PGAP was added at the arrow to yield the indicated concentrations and optical density (arbitrary units) was recorded. (a) Human platelets; (b) sheep platelets.

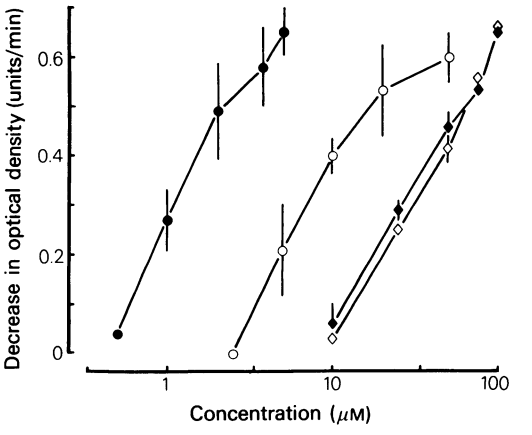


Figure 2 Log concentration-effect curves for the initial rate of aggregation of human platelets induced by ADP (●), PGAP (○), LGAP (◆) and OGAP (◇). Aggregation in PRP was monitored as described in the legend to Figure 1 and quantitated by measuring the initial rate of decrease in optical density. Symbols represent means of 3–10 assays; s.e.means shown by vertical lines.

Results

Acetal phosphatidic acid-induced human platelet aggregation and release

Each of the three acetal phosphatidic acids induced a rapid increase in the optical density of PRP, indicative of platelet shape change, which was followed by concentration-dependent aggregation of the platelets. Responses to PGAP are shown in Figure 1a and are typical of the responses to LGAP and OGAP. At threshold concentrations of the three agents only a reversible shape change occurred; at higher concentrations single phase aggregation followed by rapid disaggregation took place. As their concentrations were increased, the acetal phosphatidic acids elicited a second phase of aggregation which progressed to produce maximum irreversible aggregation and at still higher concentrations they induced monophasic, irreversible aggregation. Concentration-effect curves for the initial rate of aggregation induced by the acetal phosphatidic acids and ADP are shown in Figure 2. The threshold dose of PGAP for aggregation was 2.5–5 μM, compared to 10 μM for both LGAP and OGAP and the EC_{50} value for PGAP was 8.5 μM. Parallelism of the concentration-effect curves indicates that the palmitaldehyde acetal was 6–7 times less potent than ADP, but 4 times more potent than the equipotent linolealdehyde and olealdehyde acetal phosphatidic acids.

When platelet release of [^{14}C]-5-HT was monitored in PRP after stimulation with PGAP at con-

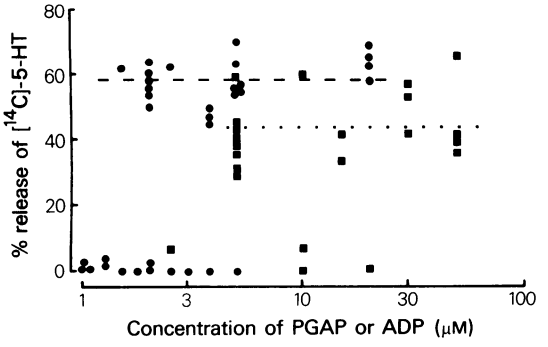


Figure 3 Dose-relationship of release of [^{14}C]-5-hydroxytryptamine ([^{14}C]-5-HT) from human platelets in response to PGAP and ADP. Release was measured in samples of [^{14}C)-5-HT-labelled platelets in PRP 4 min after initiation of aggregation with PGAP (■) or ADP (●), and is expressed as a percent of initial platelet ^{14}C content. Each symbol represents the mean of duplicate measurements of one assay. Details are described under Methods. Lines are drawn at the level of the mean percentage release for all concentrations of PGAP and ADP which were associated with second phase aggregation: PGAP, 44.0 ± 2.4% (n = 18); ADP, 58.1 ± 1.4% (n = 21).

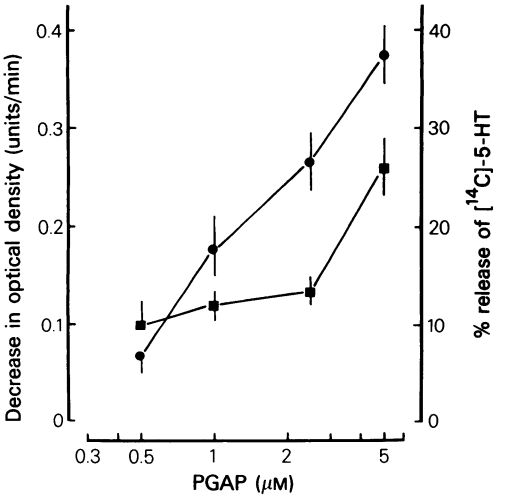


Figure 4 Log concentration-effect curves for the initial rate of aggregation of sheep platelets in PRP (●), and for [^{14}C]-5-HT release (■) from [^{14}C]-5-HT-labelled sheep platelets in PRP. See legends to Figures 2 and 3 for further details. Measurements were made using a single preparation of PRP, and are typical of results obtained with two preparations. Symbols indicate means of triplicate assays; s.e.means shown by vertical lines.

centrations which elicited second phase aggregation, the acetal induced 30–60% (44.0 ± 2.4%, n = 18) release of platelet [^{14}C]-5-HT (Figure 3). Release was not concentration-dependent, but all-or-none, and PGAP concentrations which induced only first phase aggregation did not cause release of platelet [^{14}C]-5-HT. As shown in Figure 3, similar results were obtained for ADP-treated platelets in PRP, although ADP generally induced more release (58.1 ± 1.4%, n = 21), than PGAP, and was effective at lower concentrations.

Effects of related phospholipids on human platelets

1-Palmityl lysophosphatidic acid elicited aggregation of human platelets in PRP; the threshold concentration was 100 μM and maximum aggregation occurred at approximately 750 μM. Lecithin, lysolecithin, and phosphatidic acid at concentrations of 50 μM and 100 μM neither induced platelet aggregation nor enhanced or inhibited PGAP- or ADP-induced aggregation.

PGAP-induced sheep platelet aggregation and release

PGAP caused dose-dependent aggregation of sheep platelets in PRP (Figures 1b, 4). The acetal was ten times more potent versus sheep than human platelets (Figure 4); its potency in sheep PRP was approximately equal to that of ADP. As with aggregation of sheep platelets induced by ADP (Gough, Maguire & Penglis, 1972; Gough *et al.*, 1978), sheep platelet aggregation mediated by PGAP was always monophasic (Figure 1b). The threshold concentration of PGAP for reversible aggregation was 0.25–0.5 μM, and irreversible aggregation occurred at PGAP concentrations > 5 μM. At concentrations of 0.5 μM to 5 μM, PGAP induced dose-dependent release of [^{14}C]-5-HT from prelabelled sheep platelets (Figure 4). In contrast, ADP at concentrations as high as 20 μM, more than twice the concentration necessary for maximum platelet aggregation, did not cause any significant release of [^{14}C]-5-HT from prelabelled sheep platelets.

Effect of drugs on PGAP-induced human platelet aggregation and release

Samples of human platelets in PRP, either unlabelled or labelled with [^{14}C]-5-HT, were incubated with selected inhibitors and stimulated with PGAP or ADP at concentrations that elicited biphasic aggregation in the absence of inhibitor. 2-Methylthio-AMP, a specific antagonist of ADP-induced platelet aggregation (Gough *et al.*, 1978; Maguire, 1981), at 1–2.5 μM, inhibited second phase aggregation and release induced by both PGAP and ADP (Table 2). As the concentration of 2-methylthio-AMP was increased to 12–25 μM, the first phase of ADP-induced aggregation was almost abolished, but, in contrast,

Table 2 Inhibitor concentrations required for abolition of PGAP- and ADP-induced human platelet responses in platelet-rich plasma

	Aggregation				*Release*	
	First-phase		*Second-phase*			
Aggregating agent*	ADP	PGAP	ADP	PGAP	ADP	PGAP
Inhibitor			*Inhibitor concentrations* (μM)**			
2-Methylthio-AMP	12–25	>100	1–2.5	1–2.5	1–2.5	1–2.5
Aspirin	NE<500	NE<500	100–500	100–500	100–500	100–500
Mepacrine	NE<50	50	2.5–5	2.5–5	2.5–5	2.5–5
Chlorpromazine	NE<100	10–20	20–50	10–20	20–50	5–10
PGE_1	NE<1	0.025–0.05	0.1–0.2	0.005–0.0125	—	0.005–0.0125

*Used in concentrations which gave a biphasic response: ADP 2–5 μM; PGAP 5–20 μM.
**Typical of at least 3 assays performed in a single preparation of PRP.
— = no observation. NE, no effect, i.e., no abolition of response.

the first phase of PGAP-induced aggregation was not inhibited by 2-methylthio-AMP at concentrations as high as 100 μM (Figure 5, Table 2).

The cyclo-oxygenase inhibitor, aspirin, at 100–500 μM, inhibited the second phase aggregation and release induced by both PGAP and ADP; at these concentrations aspirin did not modify first phase aggregation induced by either PGAP or ADP (Table 2, Figure 6).

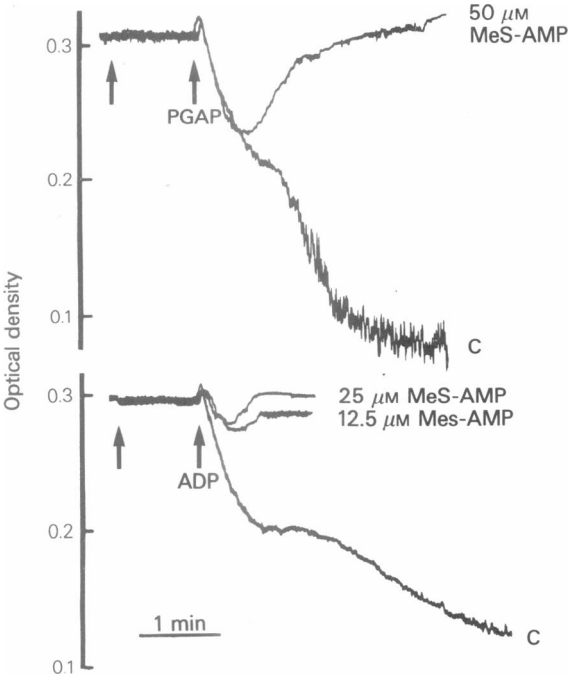


Figure 5 Comparison of the effect of 2-methylthio-AMP on PGAP and ADP-induced human platelet aggregation. 2-Methylthio-AMP (MeS-AMP) or saline (control, C) were added at the first arrow to samples of human PRP stirred in the aggregometer; after 1 min 20 μM PGAP or 2.5 μM ADP (final concentrations) were added. Optical density (arbitrary units) was recorded.

Mepacrine, a phospholipase A_2 inhibitor (Vargaftig & Dao-Hai, 1972; Vigo, Lewis & Piper, 1980), at concentrations as low as 2.5–5 μM, antagonized the second phase of PGAP- and ADP-induced platelet aggregation and release (Table 2, Figure 7). The first phase of PGAP-induced aggregation was inhibited by mepacrine in a concentration-dependent manner and was essentially abolished at 50 μM mepacrine. In contrast, the first phase of ADP-induced aggregation was only marginally sensitive to mepacrine and significant aggregation persisted at 50 μM (Table 2, Figure 7).

Prostaglandin E_1 (PGE_1), at 5–12 nM, inhibited second-phase aggregation and release induced by PGAP (Table 2, Figure 8). First-phase aggregation was abolished at 25–50 nM PGE_1 (Figure 8), and at 100 nM PGE_1 PGAP-induced shape change was completely inhibited (data not shown). ADP-induced second phase aggregation and release were much less sensitive to inhibition by PGE_1, and first phase aggregation was not completely abolished by 1 μM PGE_1.

Chlorpromazine, at concentrations of 2.5–10 μM, antagonized [^{14}C]-5-HT release and the second phase of aggregation induced by PGAP (Table 2, Figure 9), and at 10–20 μM abolished first phase aggregation. However, while 20–50 μM chlorpromazine abolished both release and second phase aggregation elicited by 5 μM ADP, the first phase of aggregation was not affected by concentrations of chlorpromazine up to 100 μM (Table 2, Figure 9).

Other drugs which inhibited PGAP-induced aggregation were reserpine, lidocaine, theophylline and

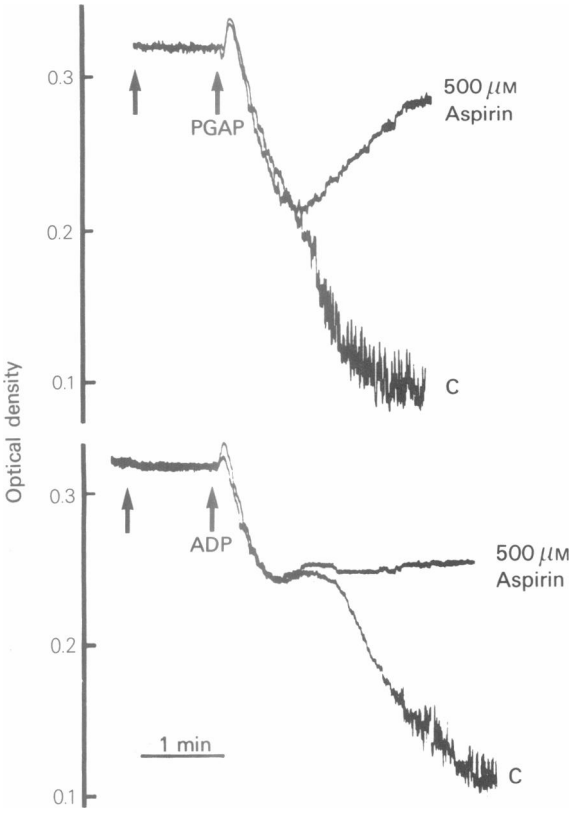


Figure 6 Comparison of the effects of aspirin on PGAP- and ADP-induced human platelet aggregation. Aspirin or solvent (control, C) were added at the first arrow to samples of human PRP stirred in the aggregometer; after 1 min 15 μM PGAP or 2 μM ADP (final concentrations) were added. Optical density (arbitrary units) was recorded.

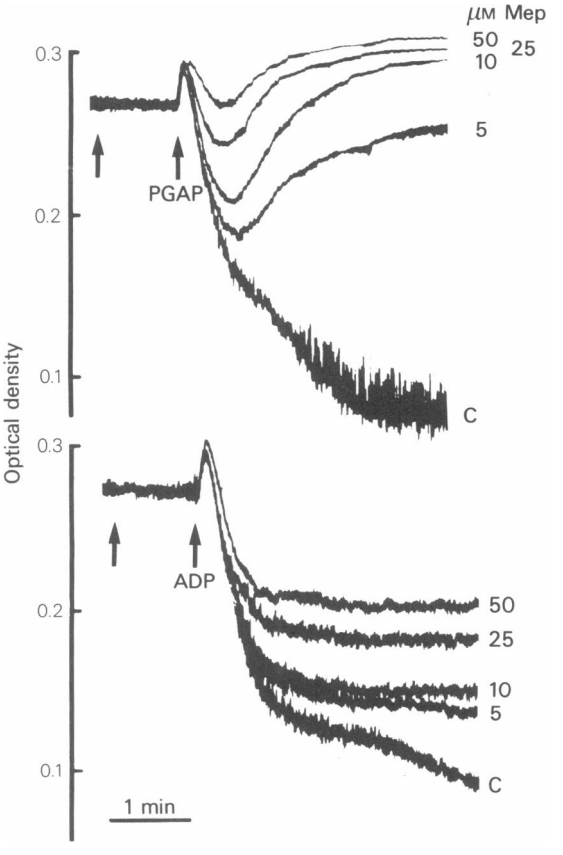


Figure 7 Comparison of the effect of mepacrine on PGAP- and ADP-induced human platelet aggregation. Mepacrine (Mep) or saline (control, C) were added at the first arrow to samples of human PRP stirred in the aggregometer; after 1 min 20 μM PGAP or 5 μM ADP (final concentrations) were added. Optical density (arbitrary units) was recorded.

methysergide; the latter two were tested as release inhibitors. Results, shown in Table 3, indicate that reserpine was the most potent of this group. Theophylline and methysergide inhibited second phase aggregation and release, but mmolar concentrations of these agents were required to abolish first phase aggregation. Lidocaine inhibited second phase aggregation but was ineffective versus first phase aggregation.

Discussion

Each of the acetal phosphatidic acids induced an immediate response in human platelets in PRP, indicating that the acetal phosphatidic acids *per se*, not their metabolites or breakdown products, were the entities that activated platelets. The acetal phosphatidic acids are stable in neutral aqueous solution at room temperature, as indicated by complete retention of platelet aggregating potency for periods up to 24 h. The palmitaldehyde derivative was the most potent of the three acetal phosphatidic acids. The unsaturated congeners, LGAP (18:2) and OGAP (18:1) were equipotent as aggregators of human platelets, implying that the degree of unsaturation of the hydrocarbon chain is not important for platelet aggregating activity. The greater potency of the saturated C_{16} congener, PGAP, suggests that length of the hydrocarbon chain may be important in optimizing platelet-stimulating activity. The non-acidic phospholipids lecithin and lysolecithin did not induce aggregation of human platelets in PRP, but l-palmityl lysophosphatidic acid was found to be a relatively weak aggregating agent, in agreement with the find-

Table 3 Inhibitor concentrations required for abolition of PGAP-induced human platelet responses*

	Aggregation		*Release*
	First-phase	*Second-phase*	
Inhibitor	*Inhibitor concentration* (μM)**		
Reserpine	NE < 30	15–20	—
Theophylline	1,000	100–150	100–150
Methysergide	1,000	200–500	200–500
Lidocaine	NE < 1,500	300–750	—

*PGAP was used at concentrations which elicited biphasic aggregation, i.e., 5–15 μM.
**Typical of 1–3 assays in 1–2 preparations of PRP.
— = no observation. NE = no effect, i.e., no abolition of response.

ings of others (Schumacher, Classen & Späth, 1979; Gerrard, Kindom, Peterson & White, 1979). Gerrard, Kindom, Peterson, Peller, Krantz & White (1979) found that l-oleyl lysophosphatidic acid also aggregated human platelets but was less active than the palmityl derivative. Phosphatidic acid, in concentrations comparable with the effective concentration of l-palmityl lysophosphatidic acid, was without aggregating activity, indicating that acylation of the 2-hydroxyl with a long chain fatty acid renders the molecule ineffective. On the other hand, platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a phospholipid in which the 2-hydroxyl is acetylated and the phosphate is esterified by choline, is a potent aggregating and releasing agent of washed rabbit platelets (De-

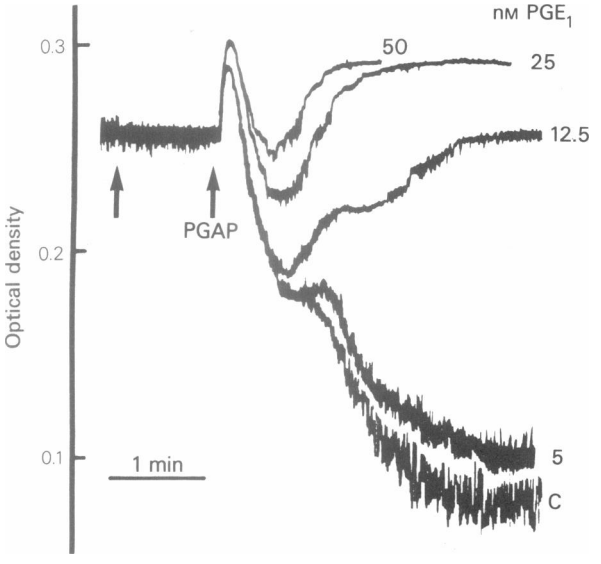


Figure 8 Effect of PGE_1 on PGAP-induced human platelet aggregation. Samples of PRP were stirred at 37°C in the aggregometer for 1 min with the indicated concentrations of PGE_1 or with saline (control, C) before challenge with 5.0 μM (final concentration) PGAP. Optical density (arbitrary units) was recorded.

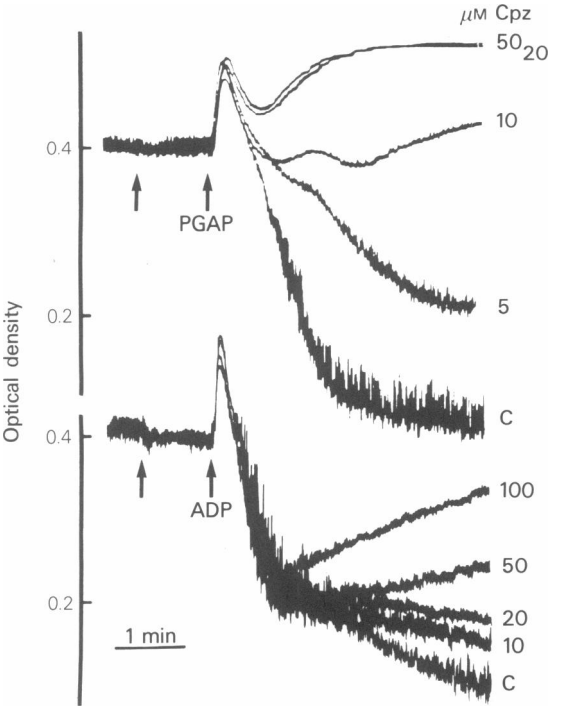


Figure 9 Comparison of the effects of chlorpromazine on PGAP- and ADP-induced human platelet aggregation. Chlorpromazine (Cpz) or saline (control, C) were added at the first arrow to samples of human PRP stirred in the aggregometer; after 1 min 10 μM PGAP or 5 μM ADP (final concentrations) were added. Optical density (arbitrary units) was recorded.

mopoulos, Pinckard & Hanahan, 1979; Cazenave, Benveniste & Mustard, 1979). The potency of PAF as an aggregating agent is strongly species-specific and is reduced by plasma. Concentrations of PAF which aggregated human platelets were 3.75 μM in the presence of plasma and 0.15 μM in its absence, compared with concentrations of 60 nM and 1 nM which aggregated rabbit platelets with and without plasma, respectively (Chignard, Vargaftig, Benveniste & Le Couedic, 1980). Thus, against human platelets in PRP, PGAP displays the same order of aggregating potency as PAF. The apparent potency of PGAP is also increased in the absence of plasma, the threshold concentration for aggregation of human platelets suspended in a modified Tyrode medium being 0.5–1 μM (Brammer & Maguire, unpublished). However PGAP and PAF appear to be different types of platelet-aggregating phospholipids. Studies with structural analogues of PAF indicated that a polar head group is necessary for platelet-stimulating activity; the deesterified derivative of PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycerol 3-phosphate, which is more analogous to the acetal phosphatidic acids than is PAF, had 1/5000 of the activity of PAF in releasing [^{14}C]-5-HT from labelled rabbit platelets (Satouchi, Pinckard, McManus & Hanahan, 1981). Nevertheless it will be of interest to test the choline and ethanolamine derivatives of PGAP, when these become available. Among compounds more closely related to the acetal phosphatidic acids, the inactivity of lysolecithin and the weak activity of 1-palmityl lysophosphatidic acid, suggest that in this group a single hydrocarbon chain and a dibasic phosphate moiety on the glycerol backbone comprise a minimum requirement for platelet activation. In the conformation obtaining for the hydrocarbon chain in the acetal linkage of the acetal phosphatidic acids, the requirements for action at the platelet membrane appear to be maximized. Moreover, the acetals have two centres of molecular asymmetry (cf. Table 1) and may therefore exist as four stereoisomers: D-*cis*, D-*trans*, L-*cis* and L-*trans*. The synthetic acetal phosphatidic acids can be expected to be a mixture of these stereoisomers and the individual diastereoisomers may differ in platelet-stimulating potency. Apropos this possibility, the enantiomer of PAF was recently shown to have only one thousandth the activity of natural PAF (Heymans, Michel, Borrel, Wichrowski, Godfroid, Convert, Tence & Benveniste, 1981). It will be important to separate the stereoisomers of PGAP and determine the relative platelet-stimulating potency of each.

Acetal phosphatidic acid-induced human platelet responses resembled those of ADP but certain features of the responses indicated that the mechanism by which these compounds act is distinct from that by which ADP acts. In particular, the disaggregation which followed aggregation of human platelets induced by low concentrations of the acetals proceeded much faster than the disaggregation associated with reversible aggregation to ADP, and was complete. Moreover, while PGAP-induced sheep platelet aggregation is monophasic, resembling that of the ADP-induced response in this species, the acetal phosphatidic acid caused dose-dependent release of [^{14}C]-5-HT, but ADP did not. In fact significant [^{14}C]-5-HT release could not be detected in sheep platelets maximally aggregated by ADP. A distinct, possibly unique mechanism appears to be involved in the action of these agents on platelets.

Investigation of the effects of selected drugs on PGAP-induced aggregation of human platelets and comparison of these effects with their actions on ADP-mediated responses provided evidence to suggest that the acetal phosphatidic acids stimulate platelets by a mechanism distinct from that of ADP. PGE_1 was a particularly effective inhibitor of PGAP-induced release, secondary and first phase aggregation, and was markedly more inhibitory versus PGAP- than ADP-induced responses. This prostaglandin stimulates platelet adenyl cyclase (Mills & Macfarlane, 1977); the resulting elevated platelet cyclic AMP causes a reduction in cytoplasmic Ca^{2+} (Käser-Glanzman, Jakobova, George & Lüscher, 1977, 1978; Käser-Glanzman, Gerber & Lüscher, 1979) and as a consequence inhibits platelet aggregation, initiation of arachidonic acid metabolism and the release reaction (Feinstein, Rodan & Cutler, 1981; Rittenhouse-Simmons & Deykin, 1981). The release of platelet granule constituents in response to many platelet stimulating agents involves the arachidonate metabolite thromboxane A_2 (TxA_2), although release via TxA_2-independent pathways has been demonstrated, e.g. in the case of thrombin, collagen (Packham, Kinlough-Rathbone, Reimers, Scott & Mustard, 1977) and PAF (Cazenave *et al.*, 1979). The sensitivity of PGAP-induced aggregation and release to inhibition by PGE_1 suggests, albeit indirectly, that both these responses are dependent on internal calcium mobilization. In this regard it is pertinent that high concentrations of the cyclic nucleotide phosphodiesterase inhibitor, theophylline, abolished first phase aggregation to PGAP.

That PGAP-induced second phase aggregation and release, but not first phase aggregation, were abolished by the cyclo-oxygenase inhibitor aspirin, indicates that PGAP-induced release requires the formation of prostaglandin endoperoxide and TxA_2 and that the first phase of PGAP-induced aggregation occurs independently of the formation of these metabolites. In support of the former contention, concentrations of PGAP which induced release in human platelet suspensions also induced the formation of the arachidonate metabolite, malondial-

dehyde (Brammer, Kerecsen & Maguire, 1982). The possibility that PGAP stimulates platelets by either directly or indirectly effecting release of platelet membrane arachidonic acid was examined by means of mepacrine, a phospholipase A_2 inhibitor (Vargaftig & Dao Hai, 1972; Blackwell, Duncombe, Flower, Parsons & Vane, 1977; Vigo *et al.*, 1980). The blockade by this drug of PGAP-induced, and ADP-induced, second phase aggregation and release lends support for prostaglandin endoperoxide and TxA_2 mediation of these events. However, at higher doses mepacrine also abolished first-phase aggregation to PGAP. Mepacrine, an amphiphilic cation, may exert a non-specific 'membrane-stabilizing' effect; this drug has a variety of actions on cell membranes which includes alteration of fluidity and inhibition of membrane ionic fluxes (Seeman, 1972); these actions may reduce the platelet membrane response to PGAP. It is noteworthy that equivalent concentrations of mepacrine did not modify the first phase of aggregation to ADP, indicating the difference between ADP and PGAP actions at the platelet membrane. The phenothiazine neuroleptics, of which chlorpromazine is the prototype, are lipophilic and also have a variety of effects on membranes (Suda, Schimizu, Maeda & Shiga, 1981). Chlorpromazine, though less potent than mepacrine, inhibited both PGAP-induced biphasic aggregation and release and, at higher concentrations, inhibited first phase aggregation. As ADP-induced first phase aggregation was not abolished by chlorpromazine, this drug also discriminated between effects of ADP and PGAP at the platelet membrane. That the mechanism involved in first phase aggregation is not the same for ADP and PGAP was demonstrated by the resistance of PGAP-induced first phase aggregation to inhibition by the ADP antagonist 2-methylthio-AMP, which abolished first phase aggregation to ADP. As with chlorpromazine, the inhibitory effects of lidocaine, methysergide and reserpine on PGAP-induced aggregation may be attributable to their local anaesthetic or 'membrane stabilizing' effects. Generally, concentration ranges for inhibition by these agents of PGAP-induced aggregation correlate with concentration ranges which have been found to be optimal for inhibition of membrane-related effects such as calcium flux, promotion of membrane expansion and displacement of calcium from membranes (Seeman, 1972).

It is interesting to note that OGAP was the most potent of the three synthetic acetal phosphatidic acids with regard to visceral smooth muscle contraction, and that PGAP was inactive (Wiley *et al.*, 1970), while, as we report here, PGAP is the most potent of the 'Darmstoff' components with regard to platelet stimulation. PGAP comprised 17% of the acetal phosphatidic acid complement of 'Darmstoff' isolated from horse intestine and 32% of that prepared from rabbit kidney (Wiley *et al.*, 1970). The platelet aggregating potencies of PGAP, LGAP and OGAP make it tempting to speculate that the compounds may be of physiological or pathological importance *in vivo*. However, although mild conditions were used to obtain the kidney extract, it must be remembered that definitive evidence is required to show that these substances occur endogenously and are not artefacts of extraction.

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