Eicosanoid/thromboxane A₂-independent and -dependent generation of lysoplasmenylethanolamine via phospholipase A₂ in collagen-stimulated human platelets

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Collagen-induced human platelet stimulation is dependent on the release of arachidonic acid (AA) from membrane phospholipid and the formation of thromboxane A₂ (TxA₂) for TxA₂-induced platelet activation. Since plasmenylethanolamine represents the single major phospholipid reservoir of AA in resting human platelets, we assessed its hydrolysis via phospholipase A₂ upon platelet stimulation with low levels of collagen by determining the generation of [³H]lysoplasmenylethanolamine via eicosanoid/TxA₂-independent and -dependent processes. Ethanolamine phospholipids in platelets were pre-labelled with [³H]ethanolamine before stimulation with either collagen or the TxA₂ mimetic U46619, in the presence or absence of BW755C, a dual inhibitor of the cyclooxygenase/lipoxygenase activities, or GR32191B, a TxA₂-receptor antagonist. Collagen stimulation promoted a marked generation of [³H]lysoplasmenylethanolamine, which was only

moderately decreased when TxA₂ synthesis or TxA₂ receptors were blocked by BW755C or GR32191B respectively. The moderate rise in [³H]lysoplasmenylethanolamine formation with U46619 as the agonist was only slightly affected by BW755C and blocked by GR32191B. Evidence for eicosanoid/TxA₂-independent and -dependent generation of [³H]lysophosphatidylethanolamine was also obtained. A significant quantitative loss of AA from plasmenylethanolamine was also demonstrated in collagen-stimulated platelets. The present findings indicate the activation of plasmenylethanolamine cleavage via phospholipase A₂ in collagen-stimulated human platelets, which, to a considerable extent, does not depend on eicosanoid/TxA₂ synthesis. This may represent an important source of releasable AA for TxA₂ generation and the promotion of further liberation of AA and phospholipid-mediated signalling pathways.

INTRODUCTION

Platelet stimulation by agonists such as collagen, thrombin or the calcium ionophore A23187 leads to the release of arachidonic acid (AA; C_{20:4,n-6}) from membrane phospholipids [1–6]. The stimulated release of AA upon receptor occupancy, via phospholipase A₂, and the subsequent metabolism of AA through the cyclooxygenase and thromboxane synthetase generates the potent pro-aggregatory metabolite thromboxane A₂ (TxA₂). Recent studies in human platelets have focused on AA turnover within the individual phospholipids [2,4,5,7–11]. Much of the research in this area has employed high levels of thrombin as an agonist which permits the monitoring of radioactivity loss from the various phospholipids after pre-labelling with AA. The latter studies have indicated that phosphatidylcholine [1,2,4,5,7,9–11] and phosphatidylethanolamine [5,9] are major sources of phospholipase A₂-mediated AA release, whereas some AA can also be freed by diacylglycerol/monoacylglycerol lipase activities after phospholipase C action on phosphoinositides [12,13]. The possible role of plasmenylethanolamine (1-*O*-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine) as a source of AA for TxA₂ synthesis has also been suggested [2,7,10,11].

Collagen-induced platelet aggregation is dependent on the early release of AA and its subsequent conversion into TxA₂ [14–18], especially at lower levels of collagen [16,18]. TxA₂ can then further promote phospholipase A₂-mediated release of AA for eicosanoid synthesis, phosphoinositide turnover and trans-

membrane signalling [6,19]. The release of AA via phospholipase A₂ is accompanied by the liberation of the corresponding 1-acyl-2-lyso-phospholipids [20]. At lower collagen levels, it has been possible to monitor the individual [¹⁴C]stearoyl-labelled 1-acyl-2-lyso-phospholipids generated via phospholipase A₂ activation under conditions where no marked decrease in the corresponding parent phospholipids was readily observed [21]. Such studies indicate that the diacylphospholipids, namely phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, can be degraded by a combination of eicosanoid-independent and -dependent processes, the TxA₂-dependent pathway being particularly active on phosphatidylcholine.

It has been determined that 45–60% of the ethanolamine-phospholipid in human platelets is represented by plasmenylethanolamine [22–24]. Since 68–72% of the fatty acids residing in the *sn*-2 position of the plasmenylethanolamine is represented by AA [22,25], this specific phospholipid is a significant contributor to the total AA-containing phospholipid in human platelets. It can be calculated that plasmenylethanolamine represents 30% of the total reservoir of AA-containing phospholipid in resting human platelets, whereas the phosphatidylcholine and phosphatidylethanolamine account for 21% and 16% respectively [22,26].

In view of the essential role of phospholipase A₂ activation and subsequent TxA₂ synthesis for platelet activation by collagen, as well as the predominance of AA-containing plasmenyl-

Abbreviations used: AA, arachidonic acid; TxA₂, thromboxane A₂; U46619, 9,11-dideoxy-11 α ,9 α -epoxy-methano-prostaglandin F_{2 α} ; BW755C, 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline; GR32191B, (1*R*)-[1 α (*Z*),2 β ,3 β ,5 α]}-(+)-7-(5-[[1,1'-biphenyl]-4-yl]methoxy)-3-hydroxy-2-(1-piperidinyl)-cyclopentyl)-4-heptenoic acid hydrochloride; ANS, 8-anilino-naphthalene-1-sulphonic acid; GPE, *sn*-glycero-3-phosphoethanolamine.

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ethanolamine in human platelets, it was decided to study the eicosanoid/TxA₂-independent and -dependent formation of lysoplasmenylethanolamine (1-*O*-alk-1'-enyl-2-lyso-*sn*-glycero-3-phosphoethanolamine) in collagen-stimulated platelets. For this purpose, ethanolamine phospholipids in platelets were pre-labelled with [³H]ethanolamine before stimulation with either collagen or the TxA₂ mimetic, U46619, in the presence and absence of either BW755C or GR32191B.

The present findings indicate that a significant portion of the lysoplasmenylethanolamine generation, resulting from the action of phospholipase A₂ on plasmenylethanolamine, occurred in an eicosanoid/TxA₂-independent manner.

MATERIALS AND METHODS

Materials

2-Amino[1-³H]ethanol hydrochloride (29.5 Ci/mmol) in aqueous solution was purchased from Amersham Canada Ltd. (Oakville, Ont., Canada). The [³H]TxB₂ radioimmunoassay kit was obtained from Du Pont-New England Nuclear (Lachine, PQ, Canada). Pre-coated t.l.c. plates (silica gel 60) were from E. Merck, Associate of BDH Chemicals Canada (Toronto, ON, Canada). Collagen was from Hormon-Chemie (München, Germany), and U46619 was supplied by Upjohn Co. (Kalamazoo, MI, U.S.A.) as a methyl acetate solution which was evaporated under nitrogen, dissolved in ethanol, and stored below 0 °C before dilution with saline. BW755C was provided by Dr. R. Ippert of the Wellcome Research Laboratories (Kirkland, PQ, Canada). GR32191B was a gift from Dr. B. M. Bain of the Glaxo Group Research Ltd. (Greenford, Middx., U.K.). Lysophosphatidylethanolamine (1-acyl-2-lyso-*sn*-glycero-3-phosphoethanolamine), and a standard mixture of plasmenylethanolamine and (diacyl)phosphatidylethanolamine, as well as 8-anilino-naphthalene-1-sulphonic acid (ANS), were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The liquid-scintillation solution CytoScint was from ICN Biomedicals (Irvine, CA, U.S.A.). All chemicals and solvents were of analytical grade.

Platelet isolation and pre-labelling

Blood was obtained by antecubital venepuncture from healthy male volunteers between 24 and 30 years of age who denied taking any medication for at least 10 days and alcohol for 2 days before the experiments. Washed platelet suspensions were prepared by the method of Mustard et al. [27], except that Hepes (5 mM final concn.) was added to the modified Tyrodes buffer. Platelets were pre-labelled in a metabolic-shaker waterbath at 37 °C for 2 h with [³H]ethanolamine (0.5 mCi/50–60 ml initial blood vol.) in the first washed platelet suspension (5 ml). Platelet counts were determined with a Coulter Counter model ZM (Coulter Electronics of Canada Ltd., Burlington, Ont., Canada) and adjusted to a final concentration of 5 × 10⁸ platelets/ml. This labelling procedure gave mean d.p.m. (all experiments) of 165 × 10³ and 379 × 10³ in the plasmenylethanolamine and phosphatidylethanolamine, respectively, per lipid extract employed for t.l.c. separation. No statistically significant alteration in the radioactivity associated with the parent phospholipids was observed (*P* > 0.05) on platelet stimulation (results not shown).

Platelet incubations

Samples (1 ml) of platelet suspensions were preincubated for 1 min in silicone-treated cuvettes with stirring at 900 rev./min,

37 °C, in an aggregometer (Payton Associates, Buffalo, NY, U.S.A.). Then 10 µl of the dual cyclo-oxygenase/lipoxygenase inhibitor [28] BW755C (100 µM final concn.), the TxA₂-receptor antagonist [29] GR32191B (1 µM final concn.), or the vehicle, water, was added 2 min before platelet stimulation with either collagen (20 µl; 2 µg/ml final concn.) or U46619 (10 µl; 1 µM). The reactions were stopped after 2 min stimulation by the rapid addition of 3.75 ml of chloroform/methanol (1:2, by vol.), and the lipids extracted.

Lipid extraction and phospholipid analyses

Lipids were extracted by the method of Bligh and Dyer [30], except that 1.25 ml of 1.8% KCl solution was used instead of water. The chloroform phase was washed with 4.75 ml of methanol/0.9% KCl (10:9, by vol.), and lipids from the initial methanol/0.9% KCl upper phase were re-extracted with 2.5 ml of chloroform. The chloroform phases were pooled, dried under nitrogen at 40 °C, and resuspended in 25 µl of chloroform/methanol (2:1, by vol.). Phospholipids were separated by two-dimensional t.l.c. by a modification of the method of Horrocks [31]. Phospholipid samples, to which 40 µg of unlabelled lysophosphatidylethanolamine standard was added, were spotted in the lower right-hand corner of silica-gel 60 plates, previously activated for 30 min at 80 °C. The plates were developed for 90 min in a paper-lined chromatographic tank, which was pre-equilibrated for 45–60 min with the first solvent consisting of chloroform/methanol/14.8 M NH₃ (130:70:11, by vol.). This first development yielded a mixed fraction containing phosphatidylethanolamine plus plasmenylethanolamine and another containing lysophosphatidylethanolamine plus lysoplasmenylethanolamine (resulting from phospholipase A₂ cleavage of plasmenylethanolamine in stimulated platelets). Between dimensions, the plates were removed, dried for 10 min at ambient temperature and then dried in an oven at 40 °C for 30 min. A 4 cm band of the silica-gel plate, containing the mixed parent and mixed lyso forms of ethanolamine-containing phospholipid, was exposed for 3 min to conc. HCl fumes by facing the plate over a Pyrex oven dish (23 cm × 14 cm) containing 100 ml of HCl. The plates were dried again at 40 °C for 15 min, before being developed from the right edge for 2 h in the second solvent system containing chloroform/methanol/88% formic acid/water (55:28:5:1, by vol.). Individual phospholipids were observed under u.v. light after spraying with 0.1% ANS solution in water [32]. The areas containing phosphatidylethanolamine, lyso(2-acyl)phosphatidylethanolamine (generated by HCl-fume exposure from plasmenylethanolamine), lysophosphatidylethanolamine, as well as GPE (generated by HCl fumes from lysoplasmenylethanolamine) were scraped into scintillation-counting vials. Both ethanolamine-containing lysophospholipids co-migrate during the first t.l.c. development; although not detected by ANS, GPE, which does not migrate during the second t.l.c. development, resides at the origin. Distilled water (1.5 ml) was added to the vials before addition of 16 ml of CytoScint, and the samples were counted for radioactivity in a liquid-scintillation counter. The measured radioactivity in the GPE fraction derived from stimulated minus control platelets represents the [³H]lysoplasmenylethanolamine resulting from phospholipase A₂ (agonist-induced) cleavage.

TxA₂ measurement

Samples (1 ml) of platelet suspensions were stimulated with collagen in the absence and presence of BW755C as described.

The reactions were stopped by addition of 25 μ M indomethacin, and the cuvettes were transferred on to ice. Platelet samples were centrifuged at 3000 *g* for 15 min at 4 °C, and the supernatants were removed and stored at -80 °C. Thromboxane B₂, the stable metabolite of TxA₂, was determined in duplicate by radioimmunoassay [33].

Mass determination of AA in ethanolamine-containing phospholipids

The AA content of the plasmenylethanolamine and phosphatidylethanolamine of resting and collagen-stimulated platelets (2 μ g/5 $\times 10^8$ platelets; 10 min) was determined by procedures similar to those previously described [25,34]. For this purpose, after t.l.c. as described above, the fatty acid methyl esters derived from the lyso(2-acyl)phosphatidylethanolamine, representing the 2-position of the parent plasmenylethanolamine, and from the phosphatidylethanolamine were analysed by g.l.c. in the presence of a known amount of pentadecanoate as an internal standard [25,34].

Statistical analysis

The data were log-transformed and analysed by analysis of variance (ANOVA). The log-transformation was not performed when ratios were to be analysed. Specific differences for pre-planned comparisons between treatment means were examined by Student's *t* test. The data of AA mass from ethanolamine-containing phospholipids were analysed by paired *t* test [35].

RESULTS

To determine the optimal time of HCl-fume exposure for complete hydrolysis of the alkenyl linkage in the plasmalogenic phospholipids (without detectable cleavage of the acyl forms), preliminary experiments were performed with a standard mixture of plasmenylethanolamine and diacylphosphatidylethanolamine from bovine brain. Maximum hydrolysis of plasmenylethanolamine occurred after 2.5 min, whereas phosphatidylethanolamine remained stable for at least 5 min upon HCl-fume treatment (results not shown). From these experiments, HCl-fume exposure was routinely set at 3 min. El Tamer et al. [36] reported some acyl-bond hydrolysis by this treatment as compared with phospholipase A₁ digestion. However, the acyl-bond hydrolysis observed in their study may be the result of a longer HCl-fume treatment (10 min).

Measurements of TxA₂ in resting and stimulated platelets exposed to collagen for 2 min gave levels of 2.8 and 69.4 ng/5 $\times 10^8$ platelets respectively, in the absence of BW755C. It was observed that more than 95 % of the agonist-induced formation of TxA₂ was blocked by preincubation with 100 μ M BW755C.

The time-dependent generation of [³H]lysoplasmenylethanolamine, via phospholipase A₂ action on [³H]plasmenylethanolamine, in collagen-stimulated platelets as studied in the absence and presence of BW755C is presented in Figure 1. The corresponding data for [³H]lysophosphatidylethanolamine generation from [³H]phosphatidylethanolamine hydrolysis are given in Figure 2. Both lysophospholipids exhibited maximal accumulation by 2 min after collagen stimulation. Preincubation of platelets with 100 μ M BW755C for 2 min before collagen stimulation only moderately decreased the formation of lysoplasmenylethanolamine (Figure 1) and lysophosphatidylethanolamine (Figure 2). Since a small decrease in the amount of accumulated lyso-

phospholipids was obtained with stimulation times extended to 3 min, routine experiments employed exposure periods of 2 min.

The combined data in Table 1 from multiple experiments show that a marked rise in [³H]lysoplasmenylethanolamine (302 % of control values) occurred upon platelet stimulation with collagen. This accumulation upon agonist exposure was only moderately decreased (to 245 % of control values) when TxA₂ synthesis was blocked by the addition of BW755C before collagen stimulation. The corresponding values for [³H]lysophosphatidylethanolamine

Figure 1 Time course showing generation of radiolabelled lysoplasmenylethanolamine in collagen-stimulated human platelets and effect of BW755C

Platelets were pre-labelled with [³H]ethanolamine and subsequently incubated (in the presence and absence of 100 μ M BW755C) for various periods of time with collagen (2 μ g/ml of platelet suspension). The radioactivity associated with the lysoplasmenylethanolamine was determined after lipid extraction and t.l.c. as described in the Materials and methods section. The data are representative (mean values) of two separate experiments. The mean radioactivity (d.p.m.) in the lysophospholipid of the unstimulated platelets was 579 and 653 in the presence and absence of BW755C respectively.

Figure 2 Time course showing generation of radiolabelled lysophosphatidylethanolamine in collagen-stimulated human platelets and effect of BW755C

Legend as for Figure 1. The mean radioactivity (d.p.m.) in the lysophospholipid of the unstimulated platelets was 2151 and 2507 in the presence and absence of BW755C respectively.

Table 1 Activation of lysophospholipid generation upon platelet stimulation with collagen or U46619 in the presence and absence of BW755C

Human platelets were pre-labelled with [^3H]ethanolamine and subsequently stimulated for 2 min with 2 μg of collagen or 1 nmol of U46619 per ml of platelet suspension (in the presence and absence of 100 μM BW755C). The radioactivity associated with the lysophospholipids (lysoplasmylethanolamine and lysophosphatidylethanolamine) was determined after lipid extraction and t.l.c. as described in the Materials and methods section. The data are given as mean $\pm$ S.E.M. from separate experiments on 4 subjects (duplicate determinations on each subject). ^{a-c}Significantly different (^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.0001$) from respective controls (without BW755C) by Student's *t* test after ANOVA analysis. ^dSignificantly different ($P < 0.0001$) from respective unstimulated controls by Student's *t* test after ANOVA analysis.

Lysophospholipid	Radioactivity in unstimulated (d.p.m.)		Stimulated (% of unstimulated values)			
			Collagen		U46619	
	— BW755C	+ BW755C	— BW755C	+ BW755C	— BW755C	+ BW755C
Lysoplasmylethanolamine	553 $\pm$ 52	516 $\pm$ 49 ^a	302 $\pm$ 4 ^d	245 $\pm$ 7 ^{c,d}	132 $\pm$ 2 ^d	129 $\pm$ 4 ^d
Lysophosphatidylethanolamine	1729 $\pm$ 227	1651 $\pm$ 233	199 $\pm$ 9 ^d	151 $\pm$ 10 ^{b,d}	135 $\pm$ 4 ^d	124 $\pm$ 9 ^d

Table 2 Comparison of radioactivity ratios (acyl species/1-alkenyl) in lysophospholipids arising from agonist exposure relative to corresponding parent phospholipids

The ratios of the net radioactivity accumulated (stimulated minus resting platelets) in the lysophospholipids due to agonist addition are given and compared with the corresponding parent phospholipids of the unstimulated platelets. The data are given as mean $\pm$ S.E.M. from separate experiments on 4 subjects (duplicate determinations on each subject). Significance by two-way analysis of variance is indicated. NS, not significantly different.

Phospholipid ratio	Unstimulated platelets			
	— BW755C		+ BW755C	
Phosphatidylethanolamine/plasmenylethanolamine	2.3 ± 0.2		2.3 ± 0.2	
	Stimulated platelets			
	Collagen		U46619	
	— BW755C	+ BW755C	— BW755C	+ BW755C
Lysophosphatidylethanolamine/lysoplasmenylethanolamine	1.5 ± 0.1	1.2 ± 0.1	3.3 ± 0.2	2.9 ± 0.2
Effect of treatments:	Collagen		U46619	
Ratios	<i>P</i> < 0.0001		<i>P</i> < 0.0006	
BW755C	NS		NS	
Interaction	NS		NS	

Table 3 Activation of lysophospholipid generation upon platelet stimulation with collagen or U46619 in the presence and absence of GR32191B

Human platelets were pre-labelled with [^3H]ethanolamine and subsequently stimulated for 2 min with 2 μg of collagen or 1 nmol of U46619 per ml of platelet suspension (in the presence and absence of 1 μM GR32191B). The radioactivity associated with the lysophospholipids (lysoplasmylethanolamine and lysophosphatidylethanolamine) was determined after lipid extraction and t.l.c. as described in the Materials and methods section. The data are given as mean $\pm$ S.E.M. from separate experiments on 4 subjects (duplicate determinations on each subject). ^aSignificantly different ($P < 0.05$) from respective controls (— GR32191B) by Student's *t* test after ANOVA analysis. ^bSignificantly different ($P < 0.0001$) from respective unstimulated controls by Student's *t* test after ANOVA analysis.

Lysophospholipid	Radioactivity in unstimulated (d.p.m.)		Stimulated (% of unstimulated values)			
			Collagen		U46619	
	— GR32191B	+ GR32191B	— GR32191B	+ GR32191B	— GR32191B	+ GR32191B
Lysoplasmylethanolamine	1127 $\pm$ 159	1033 $\pm$ 110	336 $\pm$ 38 ^b	234 $\pm$ 15 ^{a,b}	142 $\pm$ 6 ^b	104 $\pm$ 5 ^a
Lysophosphatidylethanolamine	3542 $\pm$ 518	3424 $\pm$ 543	200 $\pm$ 20 ^b	159 $\pm$ 9 ^b	150 $\pm$ 10 ^b	111 $\pm$ 7 ^a

accumulation resulting from collagen activation were 199% (— BW755C) and 151% (+ BW755C) of control values respectively (Table 1). A moderate rise to only 32% above control

values in [^3H]lysoplasmylethanolamine formation was obtained with U46619 as the agonist; this was only slightly affected by preincubation with BW755C (Table 1). The moderate

Table 4 Loss of AA from ethanolamine-containing phospholipids upon platelet stimulation with collagen

Human platelets were stimulated with collagen, and the mass determinations of AA in the plasmenylethanolamine and phosphatidylethanolamine were performed by combined t.l.c. plus g.l.c. analyses as described in the Materials and methods section. The data are given as means $\pm$ S.E.M. for AA amounts in the ethanolamine-containing phospholipids from separate experiments on 4 subjects (duplicate determinations on each subject). *Significantly different ($P < 0.05$) from unstimulated control platelets by paired *t* test.

Phospholipid	Unstimulated (nmol/ 5×10^8 platelets)	Stimulated (% of unstimulated)
Plasmenylethanolamine	17.3 ± 0.5	$95.8 \pm 1.0^*$
Phosphatidylethanolamine	11.7 ± 0.4	$93.5 \pm 1.7^*$

increase in [^3H]lysophosphatidylethanolamine with U46619 to 35% above control values was also little affected by BW755C (Table 1).

Determination of the ratios for [^3H]lysophosphatidylethanolamine/[^3H]lysoplasménylethanolamine which resulted from collagen stimulation (agonist-induced) gave a mean value of 1.5 (in the absence of BW755C), which was lower than that in the corresponding parent phospholipids (2.3) of resting platelets (Table 2). The lysophospholipid ratio was even smaller in the presence of BW755C (1.2), and approximately half that of the corresponding parent phospholipids. The use of U46619 alone as an agonist gave a corresponding ratio in lysophospholipid of 3.3; this value was not significantly affected by preincubation with BW755C.

Similar results to those observed with BW755C were obtained in collagen-stimulated platelets using the TxA_2 -receptor antagonist GR32191B (Table 3). A 325% and 234% rise in [^3H]lysoplasménylethanolamine, and a 200% and 159% rise in [^3H]lysophosphatidylethanolamine, above control values was observed in the presence or absence of GR32191B respectively. A moderate increase of 42% and 50% above control values for corresponding lysophospholipids occurred in platelets stimulated with U46619, whereas this was blocked by the presence of GR32191B.

To demonstrate that AA was lost from the ethanolamine-containing phospholipids upon low-level collagen exposure, mass determinations were performed (Table 4). The results indicated a significant ($P < 0.05$) net loss of AA from the plasmenylethanolamine (by 0.74 ± 0.19 nmol/ 5×10^8 platelets) and the phosphatidylethanolamine (by 0.74 ± 0.17 nmol/ 5×10^8 platelets) due to agonist stimulation.

DISCUSSION

As pointed out by Kambayashi et al. [11], limited information is available on the stimulus-linked metabolism of plasmenylethanolamine in platelets, owing to the relatively poor labelling of this ether phospholipid with radioactive AA, and the need to resolve the 1-alkenyl from the corresponding 1-acyl sub-class of ethanolaminephospholipid. Such labelling studies have demonstrated a marked rise in radioactive AA appearing in plasmenylethanolamine with the use of thrombin [2,4,5,7,9], a strong agonist, but do not provide direct evidence of plasmenylethanolamine breakdown via phospholipase A_2 . The latter findings have provided evidence for the transacylation pathway which mediates the transfer of AA from phosphatidylcholine to plasmenylethanolamine [10]. A corresponding decrease in the

mass of plasmenylethanolamine has been reported [37] in human platelets exposed to thrombin, as has the accumulation of lysoplasménylethanolamine in human [3] and rabbit [38] platelets. With low levels of collagen as an agonist (i.e. $2 \mu\text{g/ml}$ of platelet suspension), extreme difficulty in detecting any measurable loss of individual labelled/unlabelled phospholipids has been indicated. The present experimental approach, which employed pre-labelling of the plasmenylethanolamine (and phosphatidylethanolamine) in intact human platelets with [^3H]ethanolamine, provided the opportunity to monitor the lysoplasménylethanolamine generated via phospholipase A_2 activity using low levels of collagen (with or without BW755C) and the TxA_2 mimetic (U46619) as agonists. Free [^3H]ethanolamine can potentially enter ethanolamine phospholipid through a combination of base exchange [39] and biosynthetic reactions *de novo* [40,41].

As reviewed by Arita et al. [42], collagen addition to platelets elicits various biochemical responses in addition to platelet aggregation, including inositol phosphate formation, intracellular Ca^{2+} mobilization, AA release and TxA_2 generation. Collagen-induced platelet responses can be distinguished by TxA_2 -independent and -dependent events, with the initial TxA_2 -independent process involving activation of phospholipase A_2 , leading to AA release and TxA_2 synthesis. The TxA_2 generated in turn activates phospholipase A_2 via transmembrane signalling processes for further AA release. The present findings indicate that collagen stimulation of human platelets activates the phospholipase A_2 -mediated generation of lysoplasménylethanolamine from plasmenylethanolamine in an eicosanoid-independent manner (Figure 1 and Table 1). We observed that 72% of the generated lysoplasménylethanolamine was maintained when eicosanoid synthesis was inhibited by the dual cyclo-oxygenase/lipoxygenase inhibitor, BW755C (Table 1). The moderate diminution in agonist-induced lysoplasménylethanolamine accumulation observed upon BW755C addition (by 28%) and the ability of U46619 to promote moderately (by 29%) its generation (Table 1) indicate some hydrolysis of plasmenylethanolamine via an eicosanoid/ TxA_2 -mediated mechanism. As observed with BW755C, the presence of $1 \mu\text{M}$ GR32191B, the TxA_2 -receptor antagonist, also had only a moderate inhibitory effect on the generation of lysoplasménylethanolamine in collagen-stimulated platelets (Table 3). This suggests that the eicosanoid-dependent breakdown of these phospholipids is mainly due to TxA_2 , the potent pro-aggregatory metabolite of AA. The corresponding data on the agonist-induced generation of labelled lysophosphatidylethanolamine (Figure 2 and Table 1) are in agreement with previous work demonstrating the involvement of both eicosanoid-independent and -dependent mechanisms for the cleavage of phosphatidylethanolamine by phospholipase A_2 in collagen-stimulated human platelets [21]. In contrast with the finding on ethanolamine phospholipids, the activated phospholipase A_2 -mediated hydrolysis of phosphatidylcholine to 1-acyl (2-lyso) derivative upon platelet stimulation with low levels of collagen (under the same conditions used herein) was found to be highly sensitive to inhibition by BW755C, indicating little eicosanoid-independent hydrolysis [21].

The radioactivity ratios (lysophosphatidylethanolamine/lysoplasménylethanolamine) in the accumulated lysophospholipids (agonist-induced) upon collagen stimulation (+ BW755C), which were smaller than the corresponding parent phospholipids (1.2 versus 2.3; Table 2), suggests some preferential hydrolysis of the plasmenylethanolamine via the eicosanoid-independent process. Conversely, the somewhat higher ratio in the lysophospholipids relative to the parent phospholipids (3.3 versus 2.3) with U46619 as agonist (Table 2) suggests a moderate preference for phosphatidylethanolamine hydrolysis via the

eicosanoid/TxA₂-dependent pathway. However, a firm conclusion is difficult in this regard, since these lysophospholipids are subject to some hydrolysis by lysoplasmalogenase/lysophospholipase activities as well as acylation/transacylation reactions [10,43]. Studies *in vitro* in disrupted resting platelets have demonstrated the presence of a phospholipase A₂ which can cleave plasmenylethanolamine [44]. Although the ethanolamine-containing phospholipid was not specifically studied, a plasmalogen-selective phospholipase A₂ (Ca²⁺-independent) has been identified [45] and recently purified [46] from dog myocardium, which acts on plasmenylcholine. A Ca²⁺-dependent phospholipase A₂ which hydrolyses plasmenylcholine has been identified and purified from sheep platelets [47]. The mass measurements (Table 4) showing a significant loss of AA from plasmenylethanolamine support a simultaneous release of AA and lysoplasmylethanolamine generation via enhanced phospholipase A₂ activity in collagen-stimulated human platelets.

The relationship of phospholipase A₂-mediated hydrolysis of plasmenylethanolamine as studied herein upon collagen stimulation to platelet aggregation, phosphoinositide turnover, Ca²⁺ mobilization and protein kinase activation remains to be studied. Interestingly, Moriyama et al. [48] have concluded that TxA₂ directly causes Ca²⁺ mobilization without further activation of phospholipase C upon collagen addition to human platelets. It is possible that the eicosanoid-independent release of AA from plasmenylethanolamine is particularly important in this regard. Also of interest is the similarity of the time course of generation of [³H]ethanolamine-containing lysophospholipids in collagen-stimulated platelets (Figures 1 and 2) with that of the time-dependent formation of TxB₂ reported by Karniguian et al. [49].

In conclusion, the present study indicates that plasmenylethanolamine, which represents the single predominant reservoir of AA-containing phospholipid in resting human platelets, is hydrolysed in collagen-stimulated platelets to a considerable extent via eicosanoid/TxA₂-independent as well as eicosanoid/TxA₂-dependent mechanisms. The eicosanoid-independent hydrolysis of plasmenylethanolamine by phospholipase A₂ may represent an important source of releasable AA for the initial TxA₂ synthesis and TxA₂-induced hydrolysis of other phospholipids via phospholipase A₂ (for further AA release) and phospholipid-mediated signalling processes.

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