Aggregation and/or oxygenated products of arachidonic acid are not required for collagen-induced deacylation of phosphatidylcholine in human platelets

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In the present study the effects of collagen on platelet aggregation and arachidonic acid (AA) mobilization, specifically from phosphatidylcholine (PC), were investigated in the presence and absence of BW755C, a selective inhibitor of cyclo-oxygenase and lipoxygenases. The inhibition of cyclo-oxygenase and lipoxygenase(s) by BW755C (75 μ M) resulted in severe impairment in collagen-induced platelet aggregation. In the presence of BW755C, the aggregation response amounted to 14, 26, 37 and 49 % of the corresponding controls (without BW755C) at 10, 25, 50 and 100 μ g of collagen respectively. On the contrary, the amount of AA released from PC, which ranged from 3.5 to 8.6 nmol/10⁹ platelets, in response to the action of collagen (10–100 μ g) remained unaffected by the presence of BW755C. Substantial amounts of non-esterified AA were detected in the free fatty acid fractions obtained from collagen-stimulated platelets in the presence as well as in the absence of BW755C. However, the presence of BW755C caused a greater accumulation of free AA (mass) and this ranged from 4 to 16 nmol, depending upon the amount of collagen. In addition, small increases in free stearic and oleic acids were observed in collagen-stimulated platelets as compared with unstimulated platelets. The amount of AA lost from PC represented 67, 80, 49 and 52 % of the free AA obtained at 10, 25, 50 and 100 μ g of collagen respectively. Our results provide evidence to suggest that the adhesion of platelets to collagen fibres may be responsible for much of the AA release from PC. Furthermore, these results demonstrate that aggregation and/or cyclo-oxygenase/lipoxygenase metabolites are not obligatory for the release of AA from PC in collagen-stimulated human platelets.

INTRODUCTION

Platelet aggregation is a fundamental reaction in haemostasis and thrombosis. Most agents that stimulate platelet aggregation also induce extensive morphological and metabolic changes in these cells. These include shape change, rapid increase in inositol phospholipid turnover, an increase in cytoplasmic Ca²⁺ levels, aggregation, secretion of granule constituents and synthesis of eicosanoids (Packham & Mustard, 1984; Holmsen, 1985; Lapetina, 1986; Lagarde, 1988). Platelet adhesion to collagen appears to be independent of arachidonic acid (AA) metabolites (Lapetina *et al.*, 1986; Rao *et al.*, 1986). On the other hand, there is overwhelming evidence from studies on cyclo-oxygenase inhibitors (indomethacin, aspirin and BW755C) that platelet aggregation induced by collagen is partially dependent upon endoperoxides and/or thromboxane A₂ (TxA₂), and the requirement for these metabolites appears to be more critical at low concentrations of collagen (Kinlough-Rathbone *et al.*, 1977, 1980; Lewis & Watts, 1982; Pollock *et al.*, 1986a; Emms & Lewis, 1986). Contrary to this, the transient increase in cytoplasmic Ca²⁺ levels observed in response to collagen stimulation has been shown to be entirely dependent upon endoperoxides/

TxA₂ (Pollock *et al.*, 1984, 1986a,b). However, the role(s) of lipoxygenase-derived AA metabolites in platelet aggregation and other platelet responses remains obscure (Roth, 1986).

Platelet aggregation and secretion in response to collagen stimulation occur in parallel with the formation of diacylglycerol (DG), phosphatidic acid, inositol phosphates and AA metabolites, and the phosphorylation of a 40 kDa protein (Lloyd *et al.*, 1973; Broekman *et al.*, 1980; Rittenhouse & Allen, 1982; Sano *et al.*, 1983; Siess *et al.*, 1983a,b; Watson *et al.*, 1985; Lapetina, 1986; Siess, 1986). In collagen-mediated platelet activation, it is postulated that the initial adhesion of a limited number of platelets to collagen first triggers the activation of phosphatidylinositol (PI)-specific phospholipase C (EC 3.1.4.10) followed by other events (Lapetina, 1986). Although inositol phosphates are rapidly formed from phosphatidyl 4,5-bisphosphate by the action of phospholipase C in collagen-stimulated platelets, whether or not these phosphates participate in the activation of phospholipase A₂ remains unknown. Generally it is believed that inositol phosphates generated during platelet stimulation promote the immediate mobilization of Ca²⁺ ions from the intracellular stores into the cytosol. It has been suggested that agonist-

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; DG, diacylglycerol; AA, arachidonic acid; TxA₂, thromboxane A₂; BW755C, 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride; MG, monoacylglycerol; FFA, free fatty acid.

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induced changes in cytoplasmic Ca^{2+} may be important in the activation of phospholipase A_2 (Lapetina, 1986; Watson *et al.*, 1986; Berridge, 1987). Phospholipase A_2 in platelets appears to be regulated by both calmodulin-dependent and -independent mechanisms (Wong & Cheung, 1979; Withnall *et al.*, 1984; Ballou & Cheung, 1985).

The level of eicosanoids formed during platelet activation is tightly controlled by phospholipases that are responsible for the mobilization of AA from membrane phospholipids. Eicosanoid precursor fatty acids in stimulated platelets are derived from DG, by the action of DG/monoacylglycerol (MG) lipases and/or from phosphatidylcholine (PC), phosphatidylethanolamine (PE) and PI through phospholipases of the A_2 type (Bills *et al.*, 1977; Bell *et al.*, 1979; Chau & Tai, 1981; McKean *et al.*, 1981; Walenga *et al.*, 1981; Billah & Lapetina, 1982; Prescott & Majerus, 1983; Smith *et al.*, 1985; Broekman, 1986; Mahadevappa & Holub, 1986, 1987). More specifically, it has been shown that phospholipids including PC, PE and PI serve as important sources of AA for the synthesis of eicosanoids in collagen-stimulated platelets (Blackwell *et al.*, 1977; Rittenhouse & Allen, 1982; Pollock *et al.*, 1986a; Takamura *et al.*, 1987; Vedelago & Mahadevappa, 1988a). In addition, it has been suggested that endoperoxides and TxA_2 formed during platelet stimulation with collagen may potentiate the release of AA from membrane phospholipids (Rittenhouse & Allen, 1982). However, we and others have recently shown that deacylation of phospholipids, particularly PC, occurs even when cyclo-oxygenase activity and the cytoplasmic Ca^{2+} rise are blocked by indomethacin, aspirin or BW755C (Pollock *et al.*, 1986a; Vedelago & Mahadevappa, 1988a,b). Collagen, therefore, appears to stimulate the liberation of AA from membrane phospholipids by mechanisms additional to, and/or independent of, intracellular Ca^{2+} and/or AA metabolites derived through cyclo-oxygenase/lipoxygenase enzymes.

The present study was undertaken to investigate the effect of collagen on the release of AA from PC using BW755C, a dual inhibitor of cyclo-oxygenase/lipoxygenase(s). We also report the relative changes that occurred in free palmitic/palmitoleic, stearic, oleic, linoleic and arachidonic acids following stimulation with collagen. Our results demonstrate that aggregation and oxygenated products of AA are not required for collagen-induced deacylation of PC.

EXPERIMENTAL

Materials

Heptadecanoic, stearic and other free fatty acids and phospholipid standards, as well as 2',7'-dichlorofluorescein, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pentadecanoic acid was obtained from Nu-Chek Prep, Inc. (Elysian, MN, U.S.A.). Apyrase preparations were kindly provided by Dr. B. J. Holub (Department of Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada). Soluble collagen preparations from equine tendons suspended in iso-osmotic glucose solution, pH 2.7, were from Hormone Chemie (Munich, Germany). BW755C [3-Amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride] was kindly donated by the Wellcome Research Laboratories (Beckenham, Kent, U.K.). An aggregometer, Model 800B

(Payton Associates, Ontario, Canada) was used to measure platelet aggregation. Precoated thin layer silica gel plates (H 60) were purchased from E. Merck (Darmstadt, Germany). All other solvents and chemicals employed were of analytical grade.

Preparation of platelet suspensions

Blood from the antecubital veins of human volunteers, who had abstained from taking any anti-inflammatory drugs such as aspirin for at least 2 weeks prior to the study, was drawn into siliconized glass vacutainer tubes containing $\frac{1}{6}$ -vol. of acid citrate dextrose (2.5 g of sodium citrate, 1.4 g of citric acid and 2 g of D-glucose dissolved in 100 ml of distilled water) and mixed gently. Citrated blood was then centrifuged (250 g, 20 min at 37 °C), and the platelet-rich plasma was transferred to polypropylene centrifuge tubes. Platelets were isolated from this plasma, washed and finally suspended in Tyrode's albumin buffer containing 2 mM- Ca^{2+} , pH 7.36, as described by the method of Mustard *et al.* (1972). Platelets were counted in a Coulter counter and their number was adjusted to $5 \times 10^8/\text{ml}$. The final platelet suspensions were stored in polypropylene tubes at 37 °C until further use.

Measurement of platelet aggregation

Aliquots (1 ml) of washed platelet suspensions containing 5×10^8 platelets were transferred to siliconized cuvettes. These platelets were then stimulated with different concentrations of collagen (10–100 μg) for 3 min in the presence or absence of BW755C, a dual inhibitor of cyclo-oxygenase and lipoxygenase(s), and aggregation was recorded.

Analyses of phospholipids and free fatty acids

Aliquots (1 ml) of platelet suspensions were routinely stimulated with collagen for 3 min at 37 °C with and without BW755C in an aggregometer. Reactions were terminated by the addition of 3.75 ml of chloroform/methanol (1:2, v/v). Lipids were immediately extracted and purified essentially by the method of Bligh & Dyer (1959).

Lipid extracts were dried under oxygen-free N_2 , reconstituted in small volumes of chloroform/methanol (2:1, v/v) and spotted on to precoated t.l.c. plates. The plates were developed in chloroform/methanol/acetone/acetic acid/water (60:34:60:10:5, by vol.) for the separation of phospholipids or in heptane/isopropyl ether/acetic acid (60:40:30, by vol.) for the separation of free fatty acids. Lipid bands on t.l.c. plates were revealed by spraying with 2',7'-dichlorofluorescein and identified with the aid of standard phospholipids and free fatty acids. Bands corresponding to PC, other phospholipids and free fatty acids were scraped and quickly transmethylated with 6% methanolic sulphuric acid. Fatty acid methyl esters were extracted with light petroleum (b.p. 35–80 °C) and analysed using a fused silica megabore column (J. W. Scientific, CA, U.S.A.) as described by Mahadevappa & Powell (1989). The AA content of PC and free fatty acid (FFA) fractions obtained from stimulated and unstimulated platelets was quantified using pentadecanoic acid and/or heptadecanoic acid as internal standards. In addition, other fatty acids in the FFA fraction were quantified similarly. Further studies revealed that 75–80% of AA added to platelet suspensions was recovered under our experimental conditions. Saturated fatty acids, e.g. stearic acid, were

virtually 100 % extractable into the chloroform phase of the Bligh & Dyer (1959) method.

RESULTS

Platelet aggregation curves obtained in response to the action of collagen at various concentrations (10, 25, 50 and 100 μg) in the absence (marked A) or presence (marked B) of BW755C, a dual inhibitor of cyclo-oxygenase/lipoxygenase(s), are presented in Fig. 1. As can be seen in this Figure, a similar degree of aggregation was apparent when platelets were stimulated with 10, 25 and 75 μg of collagen in the absence of BW755C, while the response was attenuated by approx. 20 % at the highest level of collagen employed (100 μg). On the other hand, platelet aggregation was markedly suppressed at all concentrations of collagen in the presence of the inhibitor (Fig. 1). Even though a notable degree of aggregation was observed following stimulation with higher concentrations of collagen (26, 37 and 49 % at 25, 50 and 100 μg respectively), this response was virtually abolished at lower concentrations (≤ 10 μg), in the presence of BW755C. Furthermore, a significant difference was noted in the amplitude of the vertical oscillations during platelet aggregation in the presence of BW755C. This suggests that the aggregates formed in the presence of collagen alone were much larger than those obtained in the presence of BW755C.

Table 1 gives the concentrations of C_{16} and C_{18} free fatty acids obtained from stimulated as well as unstimulated human platelets. The mean concentrations of palmitic/palmitoleic ($C_{16:0}/C_{16:1}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids in unstimulated platelets were 9.9, 4.9, 4.6 and 0.8 nmol respectively. Following stimulation with collagen, a small increase occurred in the levels of stearic and oleic acids, while the levels of palmitic/palmitoleic and linoleic acids remained unchanged. The absolute amounts of stearic and oleic acids released in response to collagen stimulation rose in a concentration-dependent manner whereas the other fatty acids remained unaffected. Although the levels of $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ were not affected by the presence of BW755C, a small decrease in control levels of $C_{16:0}/C_{16:1}$ was observed.

Table 2 gives the amounts of free AA (eicosanoid precursor) obtained from stimulated and unstimulated platelets. Our results showed that unstimulated platelets

Fig. 1. Effect of BW755C on collagen-induced platelet aggregation

Platelet suspensions ($5 \times 10^8/\text{ml}$) were stirred at 37°C in an aggregometer (Payton's Associates) for 1 min with or without BW755C (75 μM) and then stimulated with different concentrations of collagen. Aggregation curves marked A and B are representative of light transmission (L.T., %) in the absence and presence of BW755C (a dual inhibitor of cyclo-oxygenase and lipoxygenase enzymes) respectively. Aggregation curves in panels (a), (b), (c) and (d) are representative of the curves obtained at 10, 25, 50 and 100 μg of collagen respectively. Aggregation curves are representative of platelet responses from three different donors.

(1×10^9 platelets) contain approx. 0.7–0.8 nmol of free AA (non-esterified). Furthermore, this basal level of free AA was not affected by the inhibition of cyclo-oxygenase/lipoxygenase(s). Following stimulation with col-

Table 1. Effect of collagen on the accumulation of palmitic/palmitoleic, stearic, oleic and linoleic acids in FFA fractions

Human platelets were stimulated with different concentrations of collagen (10–100 $\mu\text{g}/\text{ml}$) in the presence and absence of BW755C. Free fatty acids were extracted, purified and analysed as described in the Experimental section. Results are expressed in nmol and represent the means $\pm$ S.E.M. obtained from three different subjects.

Collagen ($\mu\text{g}/\text{ml}$)	Free fatty acid (nmol)							
	$C_{16:0}/C_{16:1}$		$C_{18:0}$		$C_{18:1}$		$C_{18:2}$	
	–BW	+BW	–BW	+BW	–BW	+BW	–BW	+BW
0	9.9 $\pm$ 3.5	4.5 $\pm$ 2.2	4.9 $\pm$ 0.4	4.1 $\pm$ 0.3	4.6 $\pm$ 0.9	3.7 $\pm$ 0.8	0.8 $\pm$ 0.5	0.5 $\pm$ 0.3
10	5.5 $\pm$ 2.1	6.5 $\pm$ 2.4	4.6 $\pm$ 0.4	4.1 $\pm$ 0.8	4.3 $\pm$ 0.5	4.6 $\pm$ 1.0	1.4 $\pm$ 0.4	0.5 $\pm$ 0.5
25	6.4 $\pm$ 3.1	6.7 $\pm$ 5.0	5.2 $\pm$ 0.5	4.6 $\pm$ 1.0	5.5 $\pm$ 1.9	5.8 $\pm$ 1.2	1.1 $\pm$ 0.9	0.7 $\pm$ 0.4
50	8.3 $\pm$ 4.6	7.8 $\pm$ 3.0	6.2 $\pm$ 1.0	5.5 $\pm$ 0.8	5.7 $\pm$ 1.6	5.8 $\pm$ 1.2	0.8 $\pm$ 0.5	1.0 $\pm$ 0.4
100	13.8 $\pm$ 3.7	9.7 $\pm$ 2.4	6.8 $\pm$ 1.3	7.0 $\pm$ 1.9	7.3 $\pm$ 2.5	7.5 $\pm$ 1.9	1.1 $\pm$ 0.5	1.3 $\pm$ 0.6

Table 2. Effect of collagen on the accumulation of free AA in stimulated human platelets

Human platelets were stimulated with various concentrations of collagen (10–100 $\mu\text{g/ml}$) in the presence and absence of BW755C. AA was extracted, purified and quantified as described in the Experimental section. Results are expressed in nmol and represent the means $\pm$ S.E.M., obtained from three separate subjects.

Treatment	[Collagen] ($\mu\text{g/ml}$) . . .	Free AA (nmol/ 10^9 platelets)				
		0	10	25	50	100
Platelets		0.7 $\pm$ 0.1	2.1 $\pm$ 0.4	3.1 $\pm$ 0.3	4.9 $\pm$ 0.6	5.4 $\pm$ 0.6
Platelets + BW755C		0.8 $\pm$ 0.2	5.2 $\pm$ 0.8	8.1 $\pm$ 1.1	11.1 $\pm$ 1.1	16.5 $\pm$ 2.6

lagen, a dramatic increase in the AA content of the FFA fraction occurred. In addition, a further 2–3-fold increase in the amount of free AA was apparent at all concentrations of collagen when BW755C was present in the incubation medium. The difference between the amounts of free AA obtained in the presence and absence of BW755C represents the quantity of this fatty acid that would have been converted to eicosanoids (approx. 70 %) since BW755C completely inhibits the conversion of AA to eicosanoids.

Table 3 gives the amounts of AA lost from PC following stimulation of platelets with collagen. The amount of AA lost from this phospholipid increased with collagen in a concentration-dependent manner, ranging from 3.5 to 8.6 nmol/ 10^9 platelets. The net quantity of AA released from PC when stimulated with collagen represented approx. 10, 18, 18 and 22 % of the total AA present in this phospholipid at 10, 25, 50 and 100 μg respectively. This loss from PC represents 50–80 % of the total AA obtained in the FFA fraction of collagen-stimulated platelets. More importantly, the mobilization of AA from PC was not affected by the inhibition of cyclo-oxygenase/lipoxygenase(s) at the concentrations of collagen employed (10–100 μg).

DISCUSSION

In the present study, we have examined the effect of collagen on the deacylation of phospholipids, specifically from PC. Recently, preliminary results regarding the effect of collagen on the mobilization of AA from labelled platelet phospholipids were reported from this laboratory (Vedelago & Mahadevappa, 1988a). The present report deals with the effect of collagen on the release of AA mass (unlabelled) from PC as well as on the net accumulation of AA mass in the FFA fraction, as de-

termined by a combination of t.l.c. and gas chromatography. More importantly, the results on collagen effects were derived from human platelets preincubated with and without BW755C, a dual inhibitor of cyclo-oxygenase and lipoxygenase(s), in order to further elucidate the potential importance of the eicosanoids formed during platelet stimulation for the release of AA from platelet PC. The advantage of using BW755C is that it does not, by itself, affect the activation of phospholipases (Mahadevappa & Holub, 1986; Vedelago & Mahadevappa, 1988a,b; Mahadevappa & Sicilia, 1988). However, this inhibitor can indirectly affect the activation of phospholipases, which may be partially or fully dependent upon AA metabolites, by blocking the activity of cyclo-oxygenase and lipoxygenase(s) (Vedelago & Mahadevappa, 1988a).

Our results regarding the effect of collagen on platelet aggregation are in general agreement with previous reports (Kinlough-Rathbone *et al.*, 1977, 1980; Lewis & Watts, 1982; Pollock *et al.*, 1986a; Emms & Lewis, 1986). The decrease in platelet aggregation observed at 100 μg of collagen is probably due to a higher ratio of collagen to platelets. The aggregation response induced by collagen (at all concentrations) was significantly impaired in the presence of BW755C. These results indicate that cyclo-oxygenase and/or lipoxygenase metabolites can contribute to as much as 90 % of platelet aggregation at low concentrations of collagen ($\leq 10 \mu\text{g}$). It appears, therefore, that these metabolites are essential for collagen-induced aggregation of human platelets. Similar results have been obtained by others when cyclo-oxygenase inhibitors were used (Kinlough-Rathbone *et al.*, 1980; Pollock *et al.*, 1986a). In contrast, it has been claimed recently that TxA_2 may not be important for the aggregation of human platelets by collagen (Emms & Lewis, 1986). In that report the authors mention, however,

Table 3. Effect of collagen on AA loss from PC of human platelets

Human platelets were stimulated with various concentrations of collagen (10–100 $\mu\text{g/ml}$) in the presence or absence of BW755C. PC was extracted, purified and AA content determined as described in the Experimental section. Results are expressed as nmol of AA lost from PC and represent the means $\pm$ S.E.M., from three separate subjects.

Treatment	[Collagen] ($\mu\text{g/ml}$) . . .	AA lost (nmol/ 10^9 platelets)			
		10	25	50	100
Platelets		3.9 $\pm$ 0.4	7.0 $\pm$ 1.2	6.9 $\pm$ 0.6	8.5 $\pm$ 1.2
Platelets + BW755C		3.5 $\pm$ 0.9	6.5 $\pm$ 1.6	5.5 $\pm$ 0.4	8.6 $\pm$ 0.1

that TxA₂ may be responsible for only as little as 20 % of the collagen-induced aggregation response.

It has been shown that the collagen-induced turnover of phosphoinositides and release of AA from membrane phospholipids are markedly suppressed in the absence of endoperoxides and TxA₂ (Rittenhouse & Allen, 1982; Siess *et al.*, 1983a). Furthermore, the collagen-induced elevation of cytoplasmic Ca²⁺ appears to be fully dependent upon the presence of AA metabolites (Pollock *et al.*, 1984, 1986a,b). As shown in Table 2, platelets contain detectable amounts of free AA (0.7–0.8 nmol) and it appears that much of this remains unmetabolized. Similar to other agonists, collagen also stimulates the release of AA from PI, PC and PE via the sequential action of DG and MG lipases and phospholipase A₂ (Blackwell *et al.*, 1977; Broekman *et al.*, 1980; Rittenhouse & Allen, 1982; Pollock *et al.*, 1986a; Takamura *et al.*, 1987; Vedelago & Mahadevappa, 1988a,b). It has been shown that the hydrolysis of DG (predominantly 1-stearoyl-2-arachidonoyl DG) involves the sequential removal of stearic acid, followed by that of AA (Chau & Tai, 1981; Prescott & Majerus, 1983). Assuming that the majority of stearic acid detected in the FFA fraction of platelets stimulated with collagen is released from PI via the DG lipase pathway, then any increase in the quantity of stearic acid in this fraction should be equivalent to the quantity of AA mobilized from PI by this pathway. In the present study the net increase of stearic acid was approx. 3 nmol, which represents about 20 % of the total AA released following stimulation with 100 µg of collagen. The lower levels of stearic acid obtained in the presence of BW755C may have resulted from decreased hydrolysis of phosphoinositides. Others have shown that the hydrolysis of phosphoinositides in collagen-stimulated platelets is highly dependent upon cyclo-oxygenase metabolites (Rittenhouse & Allen, 1982; Siess *et al.*, 1983a; Watson *et al.*, 1985). Our results on stearic acid levels suggest that the DG lipase pathway may contribute as much as 3.0 nmol of AA towards the synthesis of eicosanoids and are consistent with previous findings from this laboratory (Vedelago & Mahadevappa, 1988a). A small decrease in C_{16:0}/C_{16:1} mass was observed following stimulation of platelets with collagen, and this decrease is likely to have occurred due to an increased oxidation of this fatty acid. On the other hand, the 2-fold increase observed in oleic acid mass is similar to that found in platelets stimulated with thrombin (Broekman *et al.*, 1981; Smith *et al.*, 1985; Mahadevappa & Holub, 1986). The small increase in free oleic acid mass observed in this study may have resulted from the hydrolysis of lysophospholipids containing this fatty acid at the *sn*-1 position of the phospholipids.

The quantity of AA detected in FFA fractions of collagen-stimulated platelets was far greater than that of any other free fatty acids. The inhibition of cyclo-oxygenase and lipoxygenase(s) by BW755C resulted in even greater accumulations of free AA. These results are similar to those obtained by others with thrombin (Smith *et al.*, 1985; Mahadevappa & Holub, 1986, 1987; Vedelago & Mahadevappa, 1988a,b). Although this study does not allow us to calculate the relative contribution of different phospholipids to the free AA pool, the amount of AA released following stimulation with 50 µg of collagen represents only 40 % of that reported by Takamura *et al.* (1987). Furthermore, only 60 % of the value reported by this group was attained when 100 µg of

collagen was used. This discrepancy may have resulted from different methodologies employed for the isolation and quantification of the phospholipid molecular species examined. However, the results from our study indicate that AA release is a highly selective event in platelets stimulated with collagen, and these results are in general agreement with previous reports (Bills *et al.* 1977; Smith *et al.*, 1985; Mahadevappa & Holub, 1986, 1987; Purdon *et al.*, 1987; Takamura *et al.*, 1987).

The actual quantity of AA lost from PC following stimulation with collagen (10–100 µg) ranged from 3.5 to 8.6 nmol. These values are similar to those reported by Takamura *et al.* (1987). A marked decrease in the absolute loss of AA from PC would have occurred in the presence of BW755C if any cyclo-oxygenase and/or lipoxygenase metabolites had been involved in the deacylation of this phospholipid. Thus, the similar losses of unlabelled AA from PC observed in the presence or absence of BW755C demonstrate that AA metabolites are not obligatory for the deacylation of this phospholipid. These mass data confirm our recent report in which it was suggested that the deacylation of PC is not dependent upon oxygenated products of AA (Vedelago & Mahadevappa, 1988a). In addition, both platelet aggregation and phosphoinositide turnover appear to be significantly impaired in the presence of BW755C at low concentrations of collagen (L. A. Piché & V. G. Mahadevappa, unpublished work). These results are consistent with previous reports in which platelet shape change, aggregation, Ca²⁺ influx and inositol lipid turnover were suggested to be dependent upon endoperoxides and TxA₂ (Rittenhouse & Allen, 1982; Siess *et al.*, 1983a; Pollock *et al.*, 1984, 1986a,b; Vedelago & Mahadevappa, 1988a). The fact that the loss of AA from PC remained the same in the presence (virtually no aggregation) as well as in the absence of BW755C (maximum aggregation) led us to conclude that the deacylation of this phospholipid can occur independently of platelet aggregation. Thus, the direct adhesion of platelets to collagen, which itself does not appear to require endoperoxide- and/or TxA₂-mediated platelet responses, may be responsible for the initial release of AA as well as the deacylation of PC. It is, therefore, likely that collagen-induced platelet responses are triggered by the initial activation of phospholipase A₂.

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