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Mobilization of arachidonic acid in collagen-stimulated human platelets

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Stimulation of platelets with collagen results in the mobilization of arachidonic acid (AA) from phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). In this study the effect of aspirin, indomethacin, BW755C and prostaglandin H_2 (PGH_2) on labelled AA release in response to varied concentrations of collagen was investigated. Our results indicate that aspirin (0.56 mM) and indomethacin (5.6 μM) not only inhibited the collagen-mediated formation of cyclo-oxygenase metabolites, but also caused a significant reduction in the accumulation of free labelled AA and 12-hydroxyeicosatetraenoic acid (12-HETE) (21–64 %) . Aspirin and indomethacin also inhibited the release of [^{3}H]AA from PC (37–75 %) and PI (33–63 %). The inhibition of AA release caused by aspirin was reversed partially by PGH_2 (1 μM). In contrast, a smaller/no inhibition of collagen-stimulated labelled AA and 12-HETE accumulation (0–11 %) and of collagen-stimulated AA loss from PC and PI was observed in the presence of BW755C. The results obtained in the presence of aspirin, indomethacin and BW755C at lower concentrations of collagen further demonstrate that AA release from PI (45–61 % inhibition at 10 μg of collagen), but not from PC, was affected by the inhibition of cyclo-oxygenase. The results obtained on the effect of PGH_2 further support that deacylation of phospholipids occurs independently of cyclo-oxygenase metabolites, particularly at higher concentrations of collagen. These results also demonstrate that aspirin and indomethacin, but not BW755C, cause a direct inhibition of collagen-induced [^{3}H]AA liberation from PC as well as from PI. We also conclude that the diacylglycerol lipase pathway is a minor, but important, route for AA release from PI in collagen-stimulated human platelets. The mechanisms underlying the regulation of AA release by collagen in the absence of cyclo-oxygenase metabolites are not clear.

INTRODUCTION

The release of arachidonic acid (AA) from membrane phospholipids is the rate-limiting step in the biosynthesis of eicosanoids. Evidence has been provided by groups from several laboratories for the existence of different pathways that may be responsible for the mobilization of AA from membrane phospholipids in stimulated platelets (Bills *et al.*, 1977; Bell *et al.*, 1979; Rittenhouse-Simmons & Deykin, 1981; McKean *et al.*, 1981; Billah *et al.*, 1981; Billah & Lapetina, 1982; Mahadevappa & Holub, 1986). Collagen is a potent stimulant of platelet responses, which include platelet shape change, aggregation, secretion, phosphoinositide turnover and Ca^{2+} mobilization from intracellular stores (Lapetina, 1986). Collagen also stimulates the release of AA from membrane phospholipids and its subsequent conversion to eicosanoids (Blackwell *et al.*, 1977). It has been suggested that deacylation of phosphatidylcholine (PC) by phospholipase A_2 (PLA_2) represents the major route for the release of AA in collagen-stimulated human platelets (Pollock *et al.*, 1986*b*). In addition, deacylation of phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) has also been known to occur in platelets stimulated by collagen (Rittenhouse & Allen, 1982; Takamura *et al.*, 1987).

It has been suggested that phospholipases of the A_2 type are regulated by calmodulin and are therefore likely to be susceptible to agonist-induced changes in intracellular Ca^{2+} levels (Wong & Cheung, 1979; Withnall *et al.*, 1984; Ballou & Cheung, 1985). In addition, the presence of PLA_2 that is capable of hydrolysing PE in the absence of Ca^{2+} has been recently reported (Ballou *et al.*, 1986). The intracellular Ca^{2+} mobilized by inositol 1,4,5-trisphosphate (IP_3), a product of the turnover of phosphoinositides, has been proposed to be involved in the activation of a calmodulin-dependent PLA_2 (Berridge, 1984; Lapetina, 1986). More importantly, it has been reported that collagen-induced increases in cytosolic Ca^{2+} are totally dependent upon the cyclo-oxygenase metabolites (Pollock *et al.*, 1984, 1986*a*,*b*). Aspirin and indomethacin are also known to cause a significant inhibition of the collagen-induced turnover of phosphoinositides and deacylation of phospholipids (Rittenhouse & Allen, 1982; Pollock *et al.*, 1986*b*). In other words, cyclo-oxygenase metabolites such as prostaglandin H_2 (PGH_2) and thromboxane A_2 (TxA_2) appear to potentiate the collagen-stimulated release of AA from membrane phospholipids (Rittenhouse & Allen, 1982). However, the inhibition of cyclo-oxygenase by indomethacin, which abolishes the elevation of intracellular Ca^{2+}, does not prevent the total deacylation of phospho-

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DG, diacylglycerol; PA, phosphatidic acid; IP_3, inositol 1,4,5-trisphosphate; AA, arachidonic acid; PLA_2, phospholipase A_2; TxA_2, thromboxane A_2; TxB_2, thromboxane B_2; 12-HETE, 12-hydroxyeicosatetraenoic acid; PGH_2, prostaglandin H_2; BW755C, 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride; PRP, platelet-rich plasma.

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lipids (Pollock *et al.*, 1986*b*). Furthermore, collagen has been shown to cause a significant mobilization of labelled AA from endogenous phospholipids in the presence of BW755C, a dual inhibitor of cyclo-oxygenase and lipoxygenase (Vedelago & Mahadevappa, 1988). Collagen, therefore, appears to stimulate liberation of AA from membrane phospholipids by mechanisms additional to and/or independent of intracellular Ca^{2+} and the metabolites of cyclo-oxygenase.

Several questions in relation to the source, the route [diacylglycerol (DG) lipase and PLA_2 pathways] and the amount of AA mobilized for eicosanoid biosynthesis following stimulation with collagen have yet to be resolved. In the present study we have investigated the effect of aspirin, indomethacin, BW755C and PGH_2 on [^{3}H]AA release from human platelet phospholipids in response to varied concentrations of collagen. We present evidence that aspirin and indomethacin interfere with the release of AA from labelled phospholipids, including PC, and this appears to be due to the inhibition of cyclo-oxygenase as well as the direct effect of the inhibitors on phospholipases. Our results further indicate that the inhibition of AA release, which occurs due to the absence of cyclo-oxygenase metabolites, can be overcome with higher concentrations of collagen.

EXPERIMENTAL

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (specific radioactivity, 94.5 Ci/mmol), EN^{3}HANCE spray for autoradiography and aquasol were from New England Nuclear Corp. (Boston, MA, U.S.A.). Kodak SB film was from the Eastman Kodak Co. (Rochester, NY, U.S.A.). Neutral and phospholipid standards, aspirin, indomethacin, and 2′,7′-dichlorofluorescein for t.l.c. were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Precoated thin-layer silica gel H plates (H60) were from E. Merck (Darmstadt, Germany). BW755C was donated by the Wellcome Research Laboratories (Beckenham, Kent, U.K.). PGH_2 was from Biomol (Plymouth Meeting, PA, U.S.A.). Soluble collagen preparations from equine tendons suspended in isotonic glucose solution, pH 2.7, were from Hormone Chemie (Munich, 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 before the study, was drawn into bags containing one-sixth volume of acid citrate dextrose (2.5 g of sodium citrate, 1.4 g of citric acid and 2 g of D-glucose in 100 ml) and mixed. Citrated blood was then centrifuged (200 *g*, 15 min at 37 °C), the platelet-rich plasma (PRP) aspirated into plastic tubes and prelabelled with [^{3}H]AA (2.5 μCi/15 ml of PRP) for 2 h at 37 °C. Labelled platelets were isolated from PRP, washed and resuspended in Tyrodes–albumin buffer containing 2 mM-Ca^{2+}, pH 7.36, by the method described by Mustard *et al.* (1972). Cells in the final suspension were counted by a coulter counter and the cell density was adjusted to 5×10^8 platelets/ml. The final platelet suspensions were stored in plastic tubes at 37 °C until use.

Experimental protocol and incubation conditions

[^{3}H]AA-labelled platelets (5×10^8 cells/ml) in siliconized cuvettes were routinely stimulated for 3 min with collagen in the presence and absence of aspirin, indomethacin and BW755C at 37 °C in an aggregometer (Payton Associates, Ontario, Canada). Before stimulation with varied concentrations of collagen, designated platelet aliquots were preincubated at 37 °C for 15 min with aspirin (0.56 mM dissolved in ethanol) or for 5 min with indomethacin (5.6 μM dissolved in ethanol) or for 5 min with BW755C (75 μM dissolved in saline). The final concentration of ethanol in the platelet suspensions was $< 0.25\%$. Controls with ethanol alone showed no significant difference in their response to collagen. In a separate set of experiments, labelled platelets pretreated with or without aspirin (0.56 mM) were stimulated with collagen and/or PGH_2 (1 μM) for 3 min. Following stimulation with collagen or PGH_2, reactions were terminated with 3.75 ml of chloroform/methanol (1:2, v/v). Experiments were completed within 1 h from the time of isolation and during which no observable shift was found in the radioactivity between phospholipids of the unstimulated platelets.

Table 1. Accumulation of [^{3}H]AA and [^{3}H]12-HETE following stimulation of human platelets with collagen

[^{3}H]AA-labelled human platelets and platelets pretreated with aspirin (0.56 mM), indomethacin (5.6 μM) and BW755C (75 μM) were stimulated with varied concentrations of collagen (10–100 μg/5×10^8 platelets). Lipids were extracted, purified, separated and analysed as described in the Experimental section. The results are expressed as means ± S.E.M. of triplicate determinations from one of the two similar experiments. *P* values for inhibition: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; *P* values for increased AA in the presence of BW755C: $\dagger P < 0.05$, and $\dagger\dagger\dagger P < 0.001$.

	Radioactivity (d.p.m./5×10^8 platelets)			
Treatment Collagen (μg)...	10	25	50	100
Collagen	3817 ± 93	4913 ± 115	5899 ± 61	6489 ± 292
Collagen + aspirin	1404 ± 60***	2831 ± 72***	3903 ± 122**	5049 ± 95***
Collagen + indomethacin	1338 ± 160***	2603 ± 34***	3955 ± 314**	5103 ± 38***
Collagen + BW755C	3395 ± 135*	6381 ± 386†	7191 ± 30†	10325 ± 154†††

Extractions and analyses of lipids

Lipids were extracted essentially by the method of Bligh & Dyer (1959). Our recovery studies indicated that from 90–95% of the radioactivity that was associated with free AA and 12-hydroxyeicosatetraenoic acid (12-HETE) was extracted into the lower phases obtained from the above lipid extraction method, whereas 90–98% of the cyclo-oxygenase metabolites remained in the upper aqueous phases (results not shown). In addition, analysis of cyclo-oxygenase metabolites extracted from the upper phases by the method of Salmon & Flower (1982) revealed that the major AA metabolite was thromboxane B_2 (TxB_2).

Extracted lipids were dried under oxygen-free nitrogen and resuspended in 1 ml of chloroform/methanol (2:1, by vol.). Appropriate standards were added to the lipid extracts to be able to visualize the individual lipid bands on t.l.c. plates. Lipid extracts were split into two and one portion was applied on to precoated t.l.c. plates and separated by two-dimensional t.l.c., employing chloroform/methanol/ammonia (65:35:5.5, by vol.) in the first dimension and chloroform/methanol/acetone/acetic acid/water (30:10:30:5:2.5, by vol.) in the second dimension. In this t.l.c.-separation method, free AA and 12-HETE comigrated as a single spot and were thus scraped off and analysed together. Since this method also failed to resolve PI from PS, the other half of the split lipid extract was separated and analysed by one-dimensional t.l.c., employing chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol.) (Mahadevappa, 1987). The radioactive bands corresponding to AA and 12-HETE, PC, PE, PS, PI, DG and phosphatidic acid (PA) were scraped off and counted by a liquid-scintillation counter (Beckman 7800). In these experiments, 1 ml aliquots of Bligh & Dyer (1959) upper phases were used to determine the radioactivity of cyclo-oxygenase metabolites (predominantly TxB_2), and the total radioactivity was then adjusted to the corresponding volumes of the upper phases.

Statistical analyses of results

Results are expressed as mean values of triplicate determinations ± S.E.M. A combination of analysis of variance (ANOVA) and least square difference was employed for paired comparison between means of platelets stimulated with collagen in the presence and absence of inhibitors.

RESULTS

Table 1 presents the net accumulation of [^{3}H]AA and/or 12-HETE that resulted upon stimulation of human platelets with collagen in the presence or absence of aspirin, indomethacin and BW755C. As seen in this table, collagen caused a significant accumulation of [^{3}H]AA and 12-HETE in the absence of the inhibitors. This indicates a considerable activation of phospholipases in response to collagen. On the other hand, the amount of [^{3}H]AA and 12-HETE was markedly attenuated in the presence of cyclo-oxygenase inhibitors such as aspirin and indomethacin and the degree of inhibition amounted to 21–64% compared with stimulated controls (no inhibitors). In contrast, a significantly lower/no inhibition of AA release (0–11%) was observed in the presence of BW755C compared with the effects of aspirin or indomethacin. Furthermore, the accumulation of radioactivity (free AA) was noticeably greater in the presence of BW755C than that observed for AA and 12-HETE in platelets stimulated by collagen (25–100 μg) with and without aspirin or indomethacin. This difference results from the inhibition of cyclo-oxygenase as well as lipoxygenase by BW755C, and the consequent appearance of the released radioactivity as free AA, which was extracted into the lower phases of the Bligh & Dyer's (1959) extraction medium.

Table 2 gives the net increases in the radioactivity of Bligh & Dyer's (1959) upper phases arising following activation of platelets by collagen in the presence or absence of the inhibitors. Almost all of the radioactivity associated with the cyclo-oxygenase metabolites appeared in Bligh & Dyer's (1959) upper phases, and the major cyclo-oxygenase metabolite formed in response to collagen was TxB_2. As seen in Table 2, the magnitude of the radioactivity increase varied with the concentration of collagen employed (1535 ± 9–3359 ± 165, expressed as d.p.m.). Aspirin, indomethacin and BW755C all severely depleted the collagen-induced accumulation of AA metabolites in Bligh & Dyer's (1959) upper phases, indicating the inhibition of cyclo-oxygenase (80–100%). However, these upper phases did carry small increases in radio-

Table 2. The effect of aspirin, indomethacin and BW755C on AA metabolites that accumulated in Bligh & Dyer's (1959) upper phases following stimulation with collagen

Upper phases from the same experiment described in Table 1 were used for determination of the radioactivity. It was found that the major metabolite in Bligh & Dyer's (1959) upper phases was TxB_2. The results shown are the mean values ± S.E.M. of triplicate determinations. *P* values are defined as in the legend to Table 1.

	Radioactivity (d.p.m./5 × 10^8 platelets)			
Treatment Collagen (μg) ...	10	25	50	100
Collagen	1535 ± 9	2535 ± 289	2749 ± 123	3359 ± 165
Collagen + aspirin	44 ± 94***	552 ± 115***	445 ± 33***	457 ± 34***
Collagen + indomethacin	50 ± 69***	224 ± 112***	307 ± 51***	389 ± 164***
Collagen + BW755C	93 ± 129***	130 ± 130***	251 ± 90***	233 ± 60***

Table 3. The net accumulation of free [^{3}H]AA and its metabolites in Bligh & Dyer's (1959) lower and upper phases following stimulation with collagen

In these experiments, free AA and 12-HETE were extracted into the lower phases when Bligh & Dyer's (1959) extraction solvents were used. Furthermore, these two lipids comigrated in the t.l.c. system employed (see the Experimental section). On the other hand, most of the cyclo-oxygenase metabolites were found in the upper phases and the major metabolite was TxB_2. The data shown were derived from the experiment described in Table 1 and represent the means ± S.E.M. of triplicate determinations. *P* values are as defined in the legend to Table 1.

	Radioactivity (d.p.m./5×10^8 platelets)			
Treatment Collagen (μg)...	10	25	50	100
Collagen	5352 ± 102	7438 ± 404	8648 ± 184	9848 ± 322
Collagen + aspirin	1448 ± 150***	3383 ± 185***	4348 ± 154***	5496 ± 130***
Collagen + indomethacin	1338 ± 160***	2827 ± 145***	4262 ± 365***	5492 ± 203***
Collagen + BW755C	3488 ± 264***	6511 ± 341	7442 ± 120	10558 ± 214

Table 4. The net release of [^{3}H]AA from labelled PC following stimulation of human platelets with collagen

Platelets prelabelled with [^{3}H]AA were stimulated in the presence and absence of inhibitors as described in the legend to Table 1. Radioactive PC was separated from total lipids and analysed as described in the Experimental section. The data shown represent the means ± S.E.M. of triplicate determinations from one of two similar experiments. *P* values are as defined in the legend to Table 1.

	Radioactivity (d.p.m./5×10^8 platelets)			
Treatment Collagen (μg)...	10	25	50	100
Collagen	3231 ± 631	5863 ± 115	7597 ± 1160	8789 ± 1105
Collagen + aspirin	1624 ± 927*	2959 ± 885*	3315 ± 141**	5087 ± 296**
Collagen + indomethacin	917 ± 141**	1483 ± 284**	2273 ± 570***	4638 ± 988**
Collagen + BW755C	2612 ± 545	5231 ± 1628	6080 ± 610	7578 ± 508

activity in the presence of aspirin or indomethacin compared with stimulated controls, and this may be due to a very small fraction of the lipoxygenase metabolite which may have been extracted into the upper phases.

Table 3 presents the sum of radioactivity that appeared as free AA and 12-HETE (lower phases) and as cyclo-oxygenase metabolites (upper phases) following stimulation by collagen in the presence or absence of the inhibitors. The net accumulated radioactivity in aspirin- or indomethacin-treated platelets was again very low compared with stimulated controls (no inhibitors). On the contrary, BW755C exerted little or no inhibitory effect on the net accumulation of free AA and its metabolites, particularly at higher concentrations of collagen (25–100 μg). However, a significant inhibition of AA release occurred in the presence of BW755C at lower concentrations of collagen (35% at 10 μg).

The results obtained on the effect of collagen and the effects that aspirin, indomethacin and BW755C had on the release of [^{3}H]AA from PC are summarized in Table 4. As seen in Table 4, stimulation of platelets with collagen alone resulted in a marked release of [^{3}H]AA from PC, implicating this phospholipid as the principal source of AA. The net loss of [^{3}H]AA from PC varied with the concentration of collagen (3231 ± 631–8789 ± 1105, expressed as d.p.m.). The release of labelled AA from PC was markedly inhibited by aspirin or indomethacin, but not by BW755C. The inhibition of AA release by aspirin or indomethacin remained surprisingly high at all concentrations of collagen (37–75%).

Collagen also caused a significant activation of phospholipase C-mediated hydrolysis of [^{3}H]PI and accumulation of [^{3}H]PA, and this was inhibited by aspirin or indomethacin. In these experiments, only 20–30% of the radioactivity lost from PI appeared in the newly formed PA (results not shown). The radioactivity that is not accounted for (referred to as unaccounted loss from PI) could be attributed to the loss of free AA via the DG lipase and/or PLA_2 pathways. In other words, the unaccounted radioactivity represents the difference between the radioactivity that is lost from PI and that which appeared in the newly-formed PA, and is presented in Table 5. Surprisingly, the concentration of collagen had very little effect on the loss of radioactivity from PI that was not accounted for by the newly-formed PA (1530 ± 380–1972 ± 146) and these results are in contrast with the effect of collagen on PC hydrolysis. This unaccounted loss from PI observed at different concen-

Table 5. The net loss of [^{3}H]AA from PI that is not accounted for in the newly-formed PA in human platelets following stimulation with collagen

The results summarized in this table are derived from the experiment described in the legend to Table 1. The net loss of radioactivity from PI that is not accounted for in the newly-formed PA represents the difference between the radioactivity lost from PI and gained in the newly-formed PA, following stimulation by collagen. The data shown represent the means ± S.E.M. of triplicate determinations from one of two similar experiments. *P* values are defined as in the legend to Table 1.

	Radioactivity (d.p.m./5 × 10^8 platelets)			
Treatment Collagen (μg)...	10	25	50	100
Collagen	1530 ± 380	1522 ± 91	1685 ± 133	1972 ± 154
Collagen + aspirin	616 ± 153*	873 ± 79***	1056 ± 84*	1363 ± 55**
Collagen + indomethacin	599 ± 236*	1044 ± 97**	1012 ± 40*	1418 ± 32**
Collagen + BW755C	836 ± 100*	1376 ± 60	1966 ± 361	1980 ± 113

Table 6. The effect of PGH_2 on the release of [^{3}H]AA from collagen-stimulated platelets pretreated with and without aspirin

Platelets prelabelled with [^{3}H]AA were treated with and without aspirin (0.56 mM) and then stimulated with collagen (10 and 100 μg) and/or PGH_2 (1 μM) for 3 min. Radioactive free AA and its metabolites, and PC, were analysed as described in the Experimental section. The results are expressed as the means ± S.E.M. of triplicate determinations from one of two similar experiments. *P* values are defined as in the legend to Table 1.

	Net accumulation of free AA + its metabolites (d.p.m./5 × 10^8 platelets)		Net loss of AA from labelled PC (d.p.m./5 × 10^8 platelets)	
Treatment Collagen (μg)...	10	100	10	100
Collagen	17174 ± 575	38261 ± 2455	16577 ± 2451	33012 ± 571
Collagen + aspirin	8963 ± 329***	10468 ± 316***	10916 ± 1460*	11505 ± 2461***
Collagen + aspirin + PGH_2	13544 ± 1123*	14806 ± 400***	13894 ± 2617	18018 ± 2359***

trations of collagen was also markedly attenuated by aspirin or indomethacin. In contrast, BW755C caused a 45% depletion in the unaccounted loss from PI only at lower concentrations of collagen (10 μg). We further noted that the sum of radioactivity lost from PI and PC as free AA in the presence or absence of inhibitors accounted for most of the radioactivity that accumulated as AA and AA metabolites in Bligh & Dyer's (1959) lower and upper phases (Table 3). It is known that inhibition of a PI response caused by aspirin can be reversed by PGH_2, a cyclo-oxygenase-derived metabolite. To verify whether the inhibition of AA release is direct or due to inhibition of cyclo-oxygenase, the effect of PGH_2 on [^{3}H]AA release occurring in response to collagen in the presence and absence of aspirin was examined (Table 6). Stimulation of platelets with PGH_2 alone resulted in a significant release of AA from PC (2839 ± 314) and, consequently, the accumulation of free AA and its metabolites (4330 ± 426 d.p.m.). As shown in Table 6, a significant inhibition of AA release from PC (35–65%) and of the accumulation of free AA and its metabolites (48–73%) occurred following pretreatment with aspirin. Although the inhibition of AA release from PC and total AA accumulation (AA and its metabolites) caused by aspirin was markedly reduced in the presence of PGH_2, we failed to obtain a complete reversal. The observed inhibition for AA release from PC was 16–45% and 21–61% for AA and its metabolites in the presence of PGH_2. However, the partial reversal of the effect of aspirin on AA release by PGH_2 appears to be the result of the direct effect of PGH_2.

DISCUSSION

In the present study we have examined the effect of aspirin, indomethacin (cyclo-oxygenase inhibitors) and BW755C (an inhibitor of cyclo-oxygenase and lipoxygenase) on collagen-induced liberation of AA from labelled human platelets. The use of radiolabelled platelets for determination of the origin and the route from which the esterified AA from membrane phospholipids is mobilized for eicosanoid biosynthesis has certain advantages as well as disadvantages. It has previously been emphasized by groups from several laboratories that the different phospholipid pools are not uniformly labelled when radioactive AA is employed. For example, the

alkenyl species of PE are very poorly labelled and as a result the loss of AA from these phospholipid pools that may occur following stimulation cannot be accurately measured (Rittenhouse-Simmons *et al.*, 1976, Rittenhouse-Simmons & Deykin, 1981; Irvine, 1982; Purdon & Smith, 1985; Mahadevappa, 1987). A significant hydrolysis of PS and the alkenyl species of PE has been shown to occur in human platelets stimulated with collagen (Takamura *et al.*, 1987). In contrast, we found no measurable loss of [^{3}H]AA from the total PE as well as from PS when stimulated with collagen (results not shown). Our failure to see the hydrolysis of PS and PE may be due to the use of radiolabelled platelets.

Our results on collagen-stimulated labelled AA release are in general agreement with previous reports (Blackwell *et al.*, 1977; Pollock *et al.*, 1986*b*). Release of labelled AA from collagen-stimulated platelets can occur from PI via the DG lipase and PLA_2 pathways and from PC and PE by the action of phospholipase A_2 (Bell *et al.*, 1979; Rittenhouse-Simmons, 1979; Blackwell *et al.*, 1977; Pollock *et al.*, 1986*b*; Takamura *et al.*, 1987). The ratio of the unaccounted loss of labelled AA from PI to PC obtained with different concentrations of collagen ranged from 0.47 to 0.22 and this indicates that PC is the major source of AA at all concentrations of collagen (10–100 μg). By assuming that 25% of the PI hydrolysis in collagen-stimulated human platelets occurs by the direct action of PLA_2, as reported previously (Rittenhouse & Allen, 1982), we calculated the loss of labelled AA that may have occurred by the sequential action of DG and monoacylglycerol lipases in our experiments and it was found to be 32% (10 μg) and 16% (25, 50, 100 μg) of the combined loss of AA from PC and PI via the action of PLA_2. This, however, does not include the contribution from the PLA_2-mediated deacylation of PE and PS as we could not assess their relative contributions due to the use of radiolabelled platelets. In other words, we may have overestimated the relative contribution of the DG lipase pathway towards the release of AA in collagen-activated human platelets. We have reported recently that the DG lipase pathway contributes 12–15% of the total AA released in thrombin-stimulated human platelets (Mahadevappa & Holub, 1986). However, PI appears to be an important source of AA at lower concentrations of collagen, and this may be applicable for thrombin as well (Guichardant & Lagarde, 1980). In addition, PI could potentially be an important source of TxA_2 (Tables 2 and 5). Compartmentalization of phospholipid substrates and their preferential utilization by cyclo-oxygenase or lipoxygenase has been suggested by several groups (Sautebin *et al.*, 1983; Pollock *et al.*, 1986*a*).

As seen by others, we also found a significant inhibition of total labelled AA release in the presence of aspirin and indomethacin, although the magnitude of the inhibition was not the same (Rittenhouse & Allen, 1982; Pollock *et al.*, 1986*b*). Furthermore, the amount of 12-HETE and free AA accumulated following stimulation with collagen was also significantly attenuated in the presence of aspirin and indomethacin (Tables 1 and 3). The reduced release of AA and its metabolites appears to be, therefore, not merely due to the effects of aspirin and indomethacin on the formation of its metabolites through the inhibition of cyclo-oxygenase (Tables 2 and 3). This is further supported by the effects of these inhibitors on the net loss of labelled AA from PC (Table 4) and PI (unaccounted AA loss, Table 5). Furthermore, the failure of PGH_2 to reverse the inhibition of AA release from PC, as well as its accumulation caused by aspirin, suggests that the release of AA from membrane phospholipids in collagen-stimulated platelets is only partially dependent and/or independent of the cyclo-oxygenase metabolites (Table 6). Our results also show a significant deacylation of phospholipids in the absence of cyclo-oxygenase metabolites, as reported previously (Pollock *et al.*, 1986*b*; Vedelago & Mahadevappa, 1988). The inhibition of PC hydrolysis seen in the presence of aspirin or indomethacin may, therefore, be due to their direct effects on phospholipases (Kaplan *et al.*, 1978; Jesse & Franson, 1979).

The results obtained on collagen-induced release of AA in the presence of BW755C differed significantly from those of aspirin and indomethacin. As no cyclo-oxygenase metabolites were detected in the presence of BW755C (Table 2), it is very unlikely that these metabolites were responsible for the observed differences in the release of AA. Our results further indicate that BW755C did not affect the liberation of labelled AA from PC (Table 4). In contrast, the unaccounted loss of AA from PI (Table 5) was significantly inhibited by BW755C at lower concentrations of collagen (10 μg). This was reflected in the total AA release (Table 3), indicating that cyclo-oxygenase metabolites derived from the AA of PI may be involved in the potentiation of AA release when the concentrations of collagen are low. This would mean that the release of AA from PI may be essential for the initial generation of small amounts of TxA_2 and this may be coupled to Ca^{2+} mobilization as proposed (Pollock *et al.*, 1984; Nakano *et al.*, 1987). The released endoperoxides and TxA_2 may then act synergistically to potentiate the release of AA as previously reported (Rittenhouse & Allen, 1982). This is consistent with the concept proposed for rat platelets that there are different stages in collagen-triggered platelet responses (Nakano *et al.*, 1987). However, an increase in the concentration of collagen abolishes the inhibition of AA loss from PI observed in the presence of BW755C (Table 5). These results demonstrate that deacylation of phospholipids occurs independently of cyclo-oxygenase metabolites at higher concentrations of collagen. However, the cyclo-oxygenase- and lipoxygenase-derived metabolites may play a significant role in stimulus-induced cellular responses, including AA metabolism (Borgeat *et al.*, 1983; Lapetina, 1986; Chang *et al.*, 1985).

It has been recently suggested that TxA_2-mediated effects may be more important in platelet shape change, secretion, aggregation, Ca^{2+} influx and inositol lipid turnover than AA liberation *per se* (Pollock *et al.*, 1984, 1986*a*,*b*; Nakano *et al.*, 1987). A direct interaction of collagen with its receptors, which does not appear to be coupled to Ca^{2+} influx, may be responsible for the bulk of the AA release and for its subsequent conversion to eicosanoids. It also appears that deacylation of phospholipids in collagen-stimulated platelets requires the presence of extracellular Ca^{2+} (Takamura *et al.*, 1987). In conclusion, we have shown that AA release, particularly from PI, but not PC, is affected by the inhibition of cyclo-oxygenase at lower concentrations of collagen. Furthermore, collagen at higher concentrations can induce the deacylation of phospholipids independently of cyclo-oxygenase and lipoxygenase metabolites. Our results also indicate that aspirin and indomethacin, but not BW755C, cause a direct inhibition of

collagen-induced AA liberation from PC and possibly from PI as well. We also conclude that the DG lipase pathway is a minor, but important, route for AA release from PI in collagen-stimulated human platelets.

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