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# Carrageenan-induced activation of human platelets is independent of phospholipase $A_2$ and of formation of thromboxanes

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Aggregation of washed rabbit platelets by thrombin and by carrageenan is accompanied by the activation of phospholipase $A_2$ and by the synthesis of thromboxanes. Accordingly, aggregation, the accompanying release reaction and the activation of phospholipase are blocked by *p*-bromophenacyl bromide and by CB 874 (2,3-dibromo (4′-cyclohexyl-3′-chloro)-phenyl-4-oxo-butyric acid), two recognized inhibitors of the enzyme. Since these two reagents also inhibit aggregation and the release reaction induced by thrombin and by carrageenan on washed human platelets, it might have been anticipated that the mechanisms of aggregation of the platelets from the two species are similar. Nevertheless, no thromboxanes $A_2$ or $B_2$, nor activation of phospholipase $A_2$ could be demonstrated with the use of carrageenan on human platelets, under conditions where thrombin was effective. It is concluded that carrageenan activates the human platelets by phospholipase $A_2$- and thromboxane $A_2$-independent mechanisms, and that the inhibitors of phospholipase $A_2$ may block platelet functions by mechanisms other than inhibition of the expected enzyme.

Carrageenans are sulphated polysaccharides which activate rabbit and human platelets (Vargaftig & Lefort 1977; Vargaftig 1977a,b; McMillan et al 1979; Kindness et al 1979). McMillan et al (1979) demonstrated that carrageenans induce secretion from human gel-filtered platelets when citrate was present. Aggregation was suppressed by drugs that elevated the intracellular levels of cyclic (c)AMP, whereas in the presence of aspirin only a secondary phase of carrageenan-induced aggregation, as well as the accompanying release of ATP, were blocked. This led to the conclusion that the secretory phase of carrageenan-induced aggregation of human platelets is dependent upon arachidonate metabolites. Those results agree partially with our own (Vargaftig 1977a,b), demonstrating a moderate and surmountable inhibition by aspirin of carrageenan-induced aggregation of rabbit platelets, as well as a stronger and less readily surmountable blockade of aggregation by the phospholipase $A_2$ inhibitor *p*-bromophenacyl bromide (Vargaftig et al 1980). Indirect evidence was thus provided that carrageenan stimulates platelet phospholipase $A_2$. We now demonstrate that, contrary to our earlier expectations, carrageenan fails to stimulate phospholipase $A_2$ of human platelets significantly, even though it does so for rabbit platelets, and is thus likely to trigger the activation of human platelets by other mechanisms.

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## MATERIALS AND METHODS

*Compounds.* The carrageenans used were: Viscarin, a gift from Marine Colloids, which is primarily *lambda*; refs. C 3889, *lambda*; C 4014, *iota*; C 1263, *kappa*, from Sigma. Two inhibitors of phospholipase $A_2$: *p*-bromophenacyl bromide (Koch Light) and CB 874 a gift from dr. E Vallee, Centre de Recherches Clin-Midy, Montpellier, France, which is 2,3-dibromo(4′-cyclohexyl-3′-chloro)-phenyl-4-oxo-butyric acid (Vallee et al 1979). Human fibrinogen (Kabi); bovine thrombin (Roche); collagen suspension (Hormon Chemie, Munich, FRG); arachidonic acid (AA), creatine phosphate (CP) and creatine phosphokinase (CPK) (Sigma), [1-$^{14}$C]AA, 56 mCi mmol$^{-1}$, (Radiochemical Centre, Amersham); aspirin (Aspegic, a soluble lysine salt provided by Laboratoires Egic, Paris); heparin (Choay, Paris); prostacyclin (gift from Dr. S. Moncada, The Wellcome Research Laboratories); indomethacin (Merck, Sharp & Dohme); salicylic acid (Prolabo).

*Platelet preparations.* Human platelets collected on a mixture of acetic acid citrate dextrose (ACD) (Aster & Jandl 1964) and heparin (20 U ml$^{-1}$), and rabbit platelets collected on sodium citrate 1 : 10 vol of a 3·8% solution were washed according to estab-

lished techniques (Ardlie et al 1970; Mustard et al 1972), and resuspended in the Tyrode solution described later. The procedures for the incorporation of $^{14}$C-AA according to Bills et al (1976) were described by Vargaftig et al (1980). After the label was incorporated into the platelet phospholipids two different protocols were used. In the first, 6 ml samples of the different platelet suspensions were challenged with the aggregating agents (carrageenans, thrombin (at 37 °C over 30 min with stirring. Suspensions were centrifuged, and the supernatant and the platelet button counted separately for the labelled AA metabolites and for the labelled phospholipids, respectively (Vallee et al 1979). In the second protocol, the platelet button and the supernatant were extracted and chromatographed on thin-layer plates to identify the phospholipid subspecies and the AA metabolites (Bills et al 1976; Vargaftig et al 1980).

*Platelet aggregation and release reaction.* Aggregation was studied in a Chrono-log aggregometer at 37 °C and 1100 rev min$^{-1}$. Experimental samples were of 0·4 ml, and 20 $\mu$l aliquots were collected at intervals for the determination of the released ATP with an automatic device (Pico ATP, Jobin Yvon, France), using the luciferine-luciferase reagents.

*Determination of thromboxanes $A_2$ and $B_2$.* Samples of platelet suspensions were challenged in the aggregometer with the aggregating agents and bioassayed upon the superfused rabbit aorta and coeliac artery strips (Vargaftig & Zirinis 1973; Bunting et al 1976). A radioimmunoassay for platelet thromboxane $B_2$ was performed as described by Chignard et al (1978).

## RESULTS AND DISCUSSION

Thrombin induced the expected reduction of the radioactivity of the rabbit platelet aggregates, indicating activation of phospholipase $A_2$ (Bills et al 1976; Vargaftig et al 1980). Viscarin was active, *lambda* and *iota* carrageenans were less effective, whereas *kappa* carrageenan was inactive (Table 1). This effect of thrombin and of Viscarin was inhibited by *p*-bromophenacyl bromide and by CB 874, which also suppressed aggregation and the release reaction (Vargaftig, 1977a,b; Vallee et al 1979; McMillan et al 1979). Viscarin and thrombin reduced the radioactivity of phosphatidylcholine and of phosphatidylinositol, whereas the relative amount of label contained in phosphatidylethanolamine was increased (see Vargaftig et al 1980). Labelled thromboxane $B_2$ was also increased, and those effects were all suppressed when the platelets were pre-treated with *p*-bromophenacyl bromide or with CB 874 (Table 2). These results seemed consistent with our previous demonstration that Viscarin promotes the release of thromboxane $A_2$ from rabbit platelets, which is inhibited by phospholipase $A_2$ inhibitors, and reinforced our suggestion (Vargaftig 1977a,b; Vargaftig & Lefort 1977) and that of McMillan et al (1979) that carrageenan stimulates platelets through the activation of phospholipase $A_2$. Similarly, Viscarin induced the aggregation of the washed human platelets when applied at and above 5 $\mu$g ml$^{-1}$, but only if fibrinogen (280 $\mu$g ml$^{-1}$) was present (Fig. 1). This platelet activation was prevented by 0·1–0·5 $\mu$M of prostacyclin, added 2 min beforehand. Moreover, aggregation, once established, was reversed, by 1 $\mu$M of prostacyclin. This inhibition validates our claim that carrageenan induces authentic platelet aggregation, liable to control by pharmacological stimulation of adenylate cyclase, which is the recognized mode of action of prostacyclin (Best et al 1977; Tateson et al 1977).

Aggregation and the release reaction were sup-

Table 1. Effect of thrombin and of carrageenans on platelet phospholipase $A_2$ activity. Rabbit or human platelets were incubated for 1 h with $^{14}$C-AA and processed as described (Vargaftig et al 1980). Aggregation was triggered with the indicated agonists, and the radioactivity of the phospholipid fraction from the platelet button was determined. Platelet activation with thrombin resulted in the expected decrease of the radioactivity. A similar effect was induced by carrageenans in the case of rabbit but not of human platelets. The number of separate assays is in parentheses. Figures are mean with s.d.

| | % of platelet radioactivity present in the phospholipid fraction after | | | | | |
|---|---|---|---|---|---|---|
| | | | Carrageenans (25 $\mu$g ml$^{-1}$) | | | |
| | Vehicle | Thrombin (2·5 U ml$^{-1}$) | Viscarin | Lambda | Iota | Kappa |
| Rabbit platelets | 94·6 s.d. 2·4 (6) | 58·2 s.d. 11 (6) | 67·5 s.d. 5 (6) | 81·6 s.d. 5 (4) | 81·9 s.d. 5 (4) | 92·9 s.d. 3 (4) |
| Human platelets | 93·1 s.d. 2 (3) | 64·5 s.d. 12 (3) | 94·7 s.d. 0·6 (3) | 94·7 s.d. 1·8 (3) | 94·2 s.d. 2 (3) | 95·2 s.d. 1 (3) |

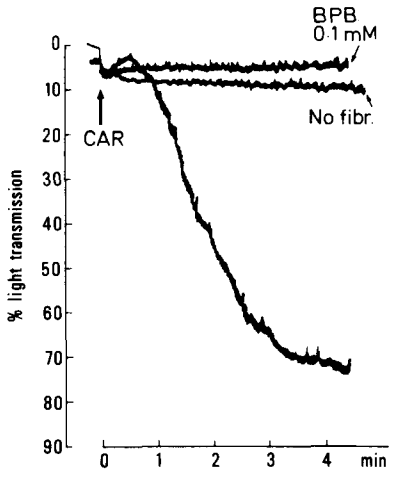


FIG. 1. Superimposed tracings of aggregation of washed human platelets induced by 10 $\mu$g ml$^{-1}$ of Viscarin (CAR) in presence and in absence of fibrinogen (fibr.). Platelets that had been in contact with bromophenacyl bromide (BPB) before further washing and resuspension in BPB-free Tyrode solution became refractory to carrageenan.

pressed by *p*-bromophenacyl bromide (50 $\mu$M) and by CB 874 (100 $\mu$M) added to the platelet suspension for 15 min before the washing procedure was continued, or directly to the platelet sample 1 min before carrageenan (Fig. 2). Since the phospholipase $A_2$ inhibitors also suppress platelet stimulation by thrombin (Vargaftig 1977; Vallee et al 1979; Vargaftig et al 1980) our results seemed to confirm the findings with the rabbit platelets and suggest

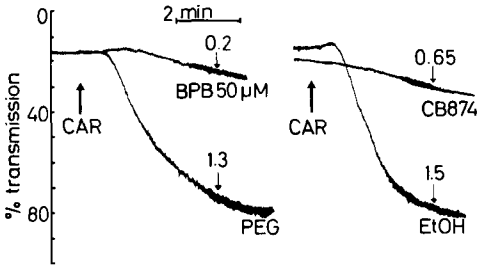


FIG. 2. Aggregation as in Fig. 1, in the presence of fibrinogen. The phospholipase $A_2$ inhibitors were added to the platelet suspension 1 min before carrageenan, in polyethyleneglycol (PEG) for BPB and in 95 °C ethanol (EtOH) for CB 874. Aggregation in controls was performed in the presence of the vehicles. Figures are the amounts (nM) of ATP released at the indicated intervals in presence and absence of the inhibitors. The high background value obtained in the presence of CB 874 results from drug interaction with the human platelet under the experimental conditions.

that phospholipase $A_2$ as necessary for the aggregation of human platelets as well. This possibility was supported by the demonstration that *p*-bromophenacyl bromide and CB 874 not only blocked phospholipase $A_2$ activity resulting from stimulation of rabbit platelets with thrombin (Vallee et al 1979; Vargaftig et al 1980), but with carrageenan as well (Table 2, representative of two similar experiments). In fact, results obtained with human platelet phospholipase $A_2$ activation differed completely from those obtained with rabbit platelets. Thus, as seen in Tables 1–4, carrageenans failed to stimulate human platelet phospholipase $A_2$ and the consequent

Table 2. Effect of thrombin and of Viscarin on radioactivity of the rabbit platelet phospholipid subspecies and on formation of thromboxane $B_2$. The figures indicate the % radioactivity present in each phospholipid subspecies compared with the total radioactivity of the whole phospholipid fraction, and the radioactivity present in thromboxane $B_2$ compared with the total radioactivity of the extract of platelet supernatant containing AA and its metabolites. The inhibitors of phospholipase $A_2$ were added to the second platelet washing solution for 15 min, under conditions described by Vargaftig et al 1980. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; $TxB_2$, thromboxane $B_2$.

| | % of the radioactivity present in the phospholipid subspecies and on thromboxane $B_2$ after | | | | |
|---|---|---|---|---|---|
| | vehicle | thrombin | Viscarin | BPB + Viscarin | CB874 + Viscarin |
| PC | 41·5 | 26·8 | 29 | 40·5 | 51·3 |
| PE | 20·3 | 35·5 | 36·1 | 21·3 | 13·4 |
| PI | 26·3 | 24·6 | 21·5 | 28·8 | 24·5 |
| $TxB_2$ | 5·7 | 14·7 | 14·5 | 4·2 | 3·1 |

release of thromboxanes (Fig. 3). The slight amounts of thromboxane $B_2$ indicated in Tables 3,4 cannot account for aggregation, and raise difficulties in interpreting the results of McMillan et al (1979), who showed aggregation of human platelets by carrageenan to be blocked by aspirin. The discrepancy may arise because aggregation according to McMillan et al (1979) required the presence of citrate, thus duplicating conditions required for the ADP-induced secondary aggregation of human platelets (Mustard et al 1975) and this is inhibited by aspirin. Another possibility is suggested by the fact that McMillan et al (1979) used gel-filtered platelets, which contain a variety of adsorbed plasma factors, whereas these factors are removed by the washing procedure we used. This accounts for our need of fibrinogen to induce aggregation, as described for ADP (Mustard et al 1972).

To clarify the role of the metabolites of AA in carrageenan-induced aggregation of human platelets,

Table 3. Failure of Viscarin to release arachidonate and thromboxane $B_2$ from human platelets. The suspension of human platelets containing $^{14}$C-AA was stimulated by thrombin (2·5 U ml$^{-1}$) and by Viscarin (25 $\mu$g ml$^{-1}$, in the presence and absence of fibrinogen, 280 $\mu$g ml$^{-1}$). The platelet button and the supernatant were extracted, and submitted to t.l.c. Values are the counts min$^{-1}$ of the phospholipids extracted from the platelet button and of the AA and thromboxane $E_2$ spots obtained from the supernatant. Thrombin-induced loss of radioactivity of the phospholipids, indicating activation of phospholipase $A_2$, confirmed by the appearance of free AA and of thromboxane $B_2$ in the supernatant. Viscarin had no overt effect of the phospholipids, and a marginal effect on free AA and thromboxane $B_2$. Abbreviations as in Table 2 and TP = total phospholipids. TAA + met = total AA + metabolites.

| | counts min$^{-1}$ of the different chemical species after platelet stimulation with | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | vehicle | % | thrombin | % | Viscarin | % | Viscarin + fibrinogen | % |
| PC | 62·450 | 46 | 36·822 | 40 | 73756 | 47 | 70117 | 52 |
| PI | 29550 | 22 | 14780 | 16 | 34485 | 22 | 26564 | 20 |
| TP | 136560 | 100 | 91240 | 100 | 156660 | 100 | 135910 | 100 |
| $TxB_2$ | 97 | 2 | 2832 | 6 | 166 | 2 | 190 | 3 |
| AA | 3500 | 70 | 24360 | 53 | 6122 | 80 | 4717 | 76 |
| + met. TAA | 5007 | 100 | 45533 | 100 | 7611 | 100 | 6160 | 00 |

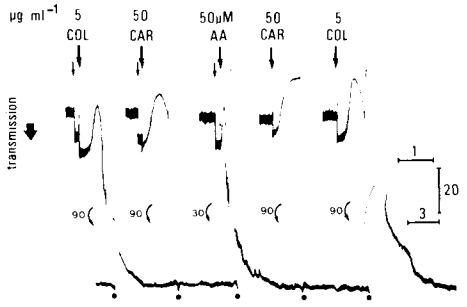


FIG. 3. Failure of Viscarin to trigger the formation of thromboxane $A_2$ by human platelets. 0·4 ml of the suspension of the human platelets were challenged in the aggregometer with the indicated amounts of collagen (COL), Viscarin (CAR) and AA (upper tracings). Fibrinogen was added before the aggregating agent when indicated by a small arrow, at the beginning of the tracing. After stirring for 90 s (except for AA, when 30 s were used), the sample was bioassayed on the superfused rabbit coeliac artery (lower tracing, each dot indicates one assay). The presence of fibrinogen accelerated aggregation by collagen, but reduced the yield of thromboxane $A_2$ activity (first and last assays); carrageenan triggered aggregation in the presence of fibrinogen (second assay) and failed to do so in its absence (fourth assay), but in both cases no thromboxane $A_2$ was detected. Aggregation was triggered by AA provided fibrinogen was present, and the expected thromboxane $A_2$ activity was detected within 30 s (third tracing). Horizontal scales: 1 min for aggregation, and 3 min for the bioassay. Vertical scale: tension of the isolated tissues in mg.

Table 4. Failure of Viscarin to release significant amounts of thromboxane $B_2$ from human platelets. 0·4 ml pf the platelet suspension were challenged in an aggregometer cuvette with Viscarin (CAR) or with thrombin, as indicated. ATP was measured within 3 min, when aggregation was completed, and sampling for the radioimmunoassay of thromboxane $B_2$ was performed within 15 min. Doses of CAR and of thrombin were 5–10 fold above those needed for aggregation. The two last experimental tubes were studied in presence of polyethyleneglycol the solvent for *p*-bromophenacyl bromide (BPB). The latter inhibited platelet aggregation and release of ATP, but less efficiently that of thromboxane $B_2$.

| Additions to platelets | Aggregation % | $TxB_2$ ng ml$^{-1}$ | ATP nM ml$^{-1}$ |
|---|---|---|---|
| fibrinogen | 1 | < 0·1 | 0·03 |
| fibrinogen + 30 $\mu$g ml$^{-1}$ CAR | 59 | 0·44 | 0·48 |
| thrombin 2·5 U ml$^{-1}$ | 54 | 37 | 0·82 |
| thrombin 0·25 U ml$^{-1}$ | 56 | 35 | 0·86 |
| PEG + fibrinogen + 30 $\mu$g ml$^{-1}$ CAR | 43 | 0·62 | 0·2 |
| BPB 50 $\mu$M + fibrinogen + 30 $\mu$g ml$^{-1}$ CAR | 1 | 0·25 | 0·02 |

aspirin, indomethacin and salicylic acid (10–50 $\mu$M) were added to the platelet suspension for 1–10 min before carrageenan. As found previously for rabbit platelets, aggregation was inhibited by the cyclooxygenase inhibitors aspirin and indomethacin, which is in agreement with McMillan et al (1979). Nevertheless, salicylic acid, which does not block cyclooxygenase, was also effective. In these experi-

ments, the platelets were stimulated with carrageenan in the presence of the potential inhibitors. We have shown previously that, under those conditions, platelet aggregation can be suppressed by a mechanism independent of inhibition of cyclo-oxygenase (Vargaftig 1977a,b). This is particularly so for salicylic acid, a very feeble inhibitor of cyclooxygenase, which may interfere with aggregation. In contrast, when the platelets are washed after being incubated with the potential inhibitors, or alternatively, when drugs are administered i.v. to rabbits before the platelets are processed, aggregation is only affected by the cyclooxygenase inhibitors (aspirin, indomethacin) and not by salicylic acid. In contrast, with blood collected from a volunteer before and 1 h after the oral administration of 0·5 g of aspirin. Platelet suspensions were prepared from the two blood samples, and aggregation studied. As expected, the effects of AA and of low amounts of thrombin were inhibited by aspirin, but aggregation by carrageenan was unaffected, even when very low doses were used (Fig. 4). Thromboxane $A_2$ was thus ruled out as a mediator of carrageenan-induced aggregation.

The other known mediator of platelet activation is ADP, the effects of which are suppressed by the combination of creatinine phosphate (CP) and creatinine phosphokinase (CPK), used to convert the released ADP into inactive ATP. This combination was effective against aggregation by carrageenan dose-dependently. Moreover, partial inhibition of aggregation by small amounts of CP/CPK was not

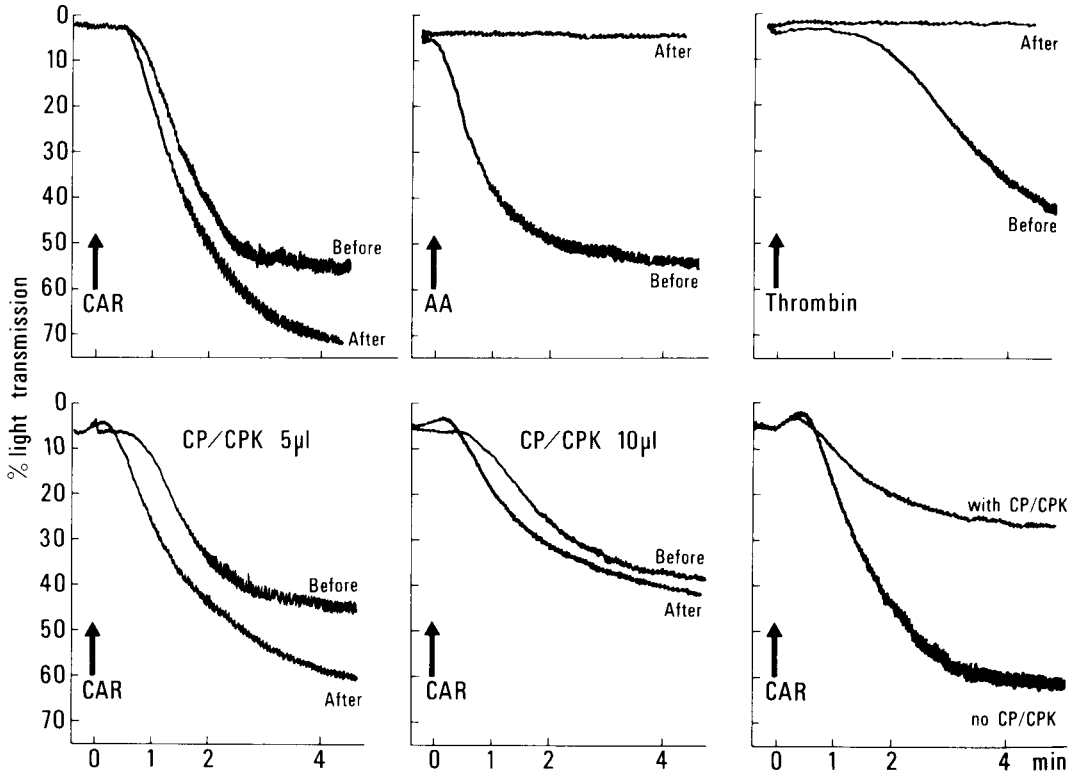


FIG. 4. Failure of aspirin and effectiveness of the ADP-scavenging system to inhibit human platelet aggregation by carrageenan. Platelet-rich plasma was prepared from citrated blood collected before and 1 h after the ingestion of 0·5 g of aspirin by a volunteer. As seen in the upper part of the Figure, aggregation by carrageenan (CAR, upper panel) was unchanged, whereas that due to arachidonic acid (AA, middle panel) and to a threshold amount of thrombin (0·025 U $ml^{-1}$, right panel) were blocked. The lower part of the Figure shows the effects of the ADP-scavenging system creatinine phosphate/creatinine phosphokinase (CP/CPK, 25 mg $ml^{-1}$ and 12 mg $ml^{-1}$, respectively) added at 5 and 10 $\mu$l (middle) to the platelets prepared from blood collected before and after the aspirin ingestion. The higher concentration of CP/CPK reduces aggregation by carrageenan, but to a similar extent for aspirin-treated or non aspirin-treated platelets. The lower right panel shows tracings obtained with platelets collected after aspirin ingestion, with and without 20 $\mu$l of CP/CPK. This amount of the scavenging system prevents aggregation by 10 $\mu$M of ADP itself (not shown).

increased when used on platelets prepared from blood collected after aspirin ingestion (Fig. 4). These results demonstrated that ADP participates in carrageenan-induced platelet aggregation, and confirmed that the small amounts of thromboxane $B_2$ detected (Tables 2–4) are not involved.

Our present results show that carrageenan does not activate human platelet phospholipase $A_2$ compared with thrombin, since (a) carrageenan failed to significantly reduce the radioactivity of platelet phospholipids and (b) it failed to significantly increase the amounts of released AA and its metabolites, thromboxanes $A_2$ and $B_2$. It might nevertheless still be argued, against our interpretation, that activation of phospholipase $A_2$ may occur at a critical site, which might explain the effectiveness of the phospholipase $A_2$ inhibitors and the slight release of AA and of its metabolites, (Tables 3,4), which is hardly comparable to that induced by thrombin or by thrombin and carrageenans on rabbit platelets. If this were so, it would provide a clear indication that the tests for phospholipase $A_2$ so far performed, including our own, only reflect very roughly the intra-platelet events. If, on the contrary, phospholipase $A_2$ is not essential for aggregation of human platelets by carrageenan, other mechanisms should be sought to account for the *p*-bromophenacyl bromide and CB 874-induced inhibition of aggregation and release reaction. Any new scheme should include the involvement of ADP with aggregation, in view of the effectiveness of CP/CPK.

We suggested previously that carrageenan activates rabbit platelet phospholipase $A_2$, leading to the release of pharmacologically active lipids other than cyclooxygenase-dependent metabolites of AA (Vargaftig 1977a). One of those lipids is "platelet-activating factor" (PAF-acether), a 1-*O*-alkyl 2 acetyl analogue of phosphatidylcholine (Demopoulos et al 1979; Benveniste et al 1979), which is formed by rabbit platelets stimulated with thrombin or with collagen (Chignard et al 1979). Formation and activity of PAF-acether are not inhibited by aspirin nor by the CP/CPK system (Chignard et al 1979; Cazenave et al 1979). The fact that scavenging ADP inhibited the effects of carrageenan suggests, but does not prove conclusively, that PAF-acether is not responsible for carrageenan-induced aggregation of human platelets.

In conclusion, we have provided evidence that carrageenan activates human platelets in the presence of fibrinogen, through a cyclooxygenase-independent and ADP-dependent mechanism, which probably does not involve phospholipase $A_2$ to the same extent as aggregation by thrombin or by collagen. Since two inhibitors of phospholipase $A_2$ prevented platelet activation by carrageenan, it appears that those inhibitors should affect platelets by other mechanisms also. One hypothesis is that deacylating enzymes involved with the membrane rearrangements occurring during the initial steps of aggregation are inhibited by drugs that suppress phospholipase $A_2$ activity. This might explain why the two irreversible inhibitors of phospholipase $A_2$ so far tested on platelets are effective against aggregation, even when it does not depend at all upon the known phospholipase $A_2$-thromboxane cascade.

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