Biochemical Pharmacology, Vol. 30, pp. 263–271.
Pergamon Press Ltd. 1981. Printed in Great Britain.

0006-2952/81/040263–09 $05.00/0

COMMENTARY

PRESENT CONCEPTS ON THE MECHANISMS OF PLATELET AGGREGATION

B. B. VARGAFTIG*, M. CHIGNARD* and J. BENVENISTE†
*Institut Pasteur, Paris, and †INSERM U 200, Clamart/Paris, France

Platelets, the smallest circulating cell fragments of mammals, are pivotal for haemostasis and for its pathological perversion, thrombosis. Platelet activation is a sequence of morphological and functional changes, with pseudopode formation, centralisation of the platelet granules due to the shortening of the contractile proteins, accompanied by the formation and secretion of a variety of substances. The primary stimulus for platelet activation appears to involve membrane glycoproteins, and receptors have been partially identified for the most studied platelet-stimulating agent, thrombin [1–4]. At a certain stage of their activation, platelets undergo rapid membrane modifications, prior to their aggregation. The latter, as opposed to agglutination, is an active process, dependent upon the platelet metabolism. Thus platelets pre-treated with formaldehyde do not aggregate, despite their reactivity to an agglutinating substance, such as ristocetin [5]. The release reaction, which may accompany aggregation, consists of the release of intragranular materials to the extracellular medium. Serotonin, ATP, ADP, calcium, and the various metabolites of arachodonic acid (AA), are thus released [6, 7].

THE FIRST PATHWAY OF AGGREGATION

Early studies suggested that ADP released from the first stimulated platelets was responsible for the further recruitment of the remaining ones, and thus for the formation of the aggregates [8]. ADP is indeed a specific platelet-stimulating agent, which induces a typical biphasic curve of aggregation when added to human, guinea-pig or beagle dog citrated platelet-rich plasma (Fig. 1). Other adenosine nucleotides are inactive, or even inhibit the effects of ADP. The role of ADP as the mediator of aggregation due to other agents, such as collagen or thrombin, is supported by its dose-dependent release during platelet stimulation, and by the reduction of platelet aggregation in the presence of scavengers of ADP [9, 10]. Aspirin, indomethacin and other non-steroidal anti-inflammatory drugs (NSAID) inhibit altogether the second wave of ADP-induced aggregation, the accompanying release reaction, and that due to small to moderate amounts of thrombin and collagen. These indirect pieces of evidence are contradicted by results obtained with thrombin-degranulated platelets, demonstrating that when

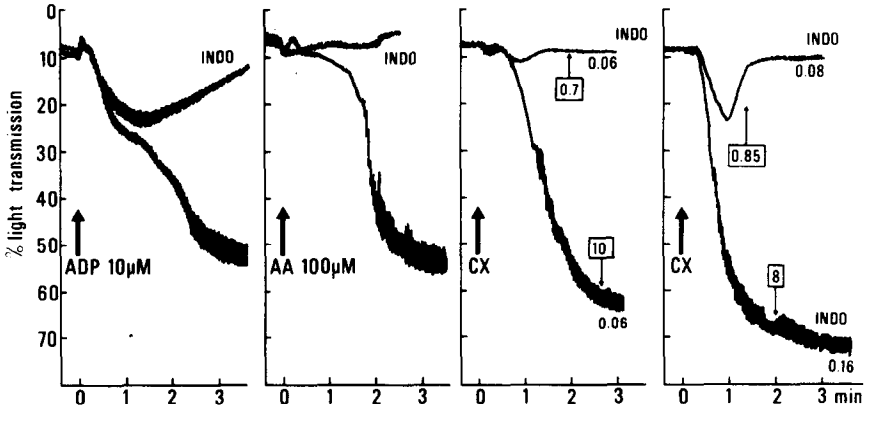

Fig. 1. Interference of indomethacin with aggregation of platelets from a beagle dog. Superimposed tracings of aggregation of beagle dog platelets, stimulated with ADP, arachidonic acid (AA), and convulxin (Cx) in the presence and in the absence of 50 μM of indomethacin as indicated. The concentrations of Cx (μg/ml) are indicated near each tracing and the amounts of ATP released (nM/ml) are inside the boxes. ADP induces a double-waved aggregation curve, which is shortcut in presence of indomethacin. Moreover, and contrary to most mongrel dogs, AA induces aggregation, which is blocked by indomethacin. Aggregation and release of ATP by the threshold amount of Cx is suppressed by indomethacin but inhibition is overcome by increasing the concentration of Cx, as shown by the tracing in the last panel where aggregation is triggered by Cx (0.16 μg/ml) despite the presence of indomethacin.

BP 30:4 - A

platelets lose the content of their granules after being challenged by large amounts of thrombin, and are recovered in appropriate conditions, both AA and ADP retain their aggregating activity, even though there is no further release of granular ADP, which has been exhausted initially [10–13]. This indicates that ADP-independent mechanisms can account for aggregation.

THE SECOND PATHWAY OF AGGREGATION

The discovery of the second pathway of aggregation, arachidonate-dependent, arose from studies performed in a completely distinct area of research. Since the early 1960's it had been shown that aspirin and other NSAID interfere with bronchoconstriction induced in the guinea-pig by different substances: "slow reacting substance of anaphylaxis", bradykinin, ATP, and AA [14]. At that time no particular importance was given to the fact that AA displayed very peculiar properties, distinct from those of parent fatty acids. Bradykinin exerts another aspirin-sensitive effect, since its hypotensive effects are curtailed by the various NSAID. One hypothesis to explain this activity was that bradykinin releases another mediator, and Piper and Vane demonstrated that such a mechanism might exist [15]. Indeed, bradykinin releases from the isolated guinea-pig lungs an unstable material, named "rabbit aorta contracting substance" (RCS), and now identified to thromboxane A2 (TxA2). Release of RCS was inhibited by NSAID. Since NSAID inhibit the hypotensive response to another "slow reacting substance" (SRS-C), released from egg yolk or tissue phospholipids by phospholipases A2 [16–17], it became clear, around 1970, that NSAID interfere at some stage with the effects of the direct administration or of the release of a specific fatty acid, namely AA. This was confirmed by the finding that AA is converted by the guinea-pig lungs into a RCS similar to that released by bradykinin. This conversion was blocked by NSAID [18]. At that time, we thought that RCS was a mixture of the endoperoxides formed during prostaglandin biosynthesis (PGG2 and PGH2), and this led to the discovery that indeed aspirin and other NSAID inhibit the synthesis of prostaglandins [19]. This was found at the same time (and independently) for the lung [19] and spleen tissues [20], on one side, and for the thrombin-stimulated platelets on the other [21]. Since we were working on the mechanisms of release of RCS by AA and by SRS-C, particularly from blood [22], we raised the hypothesis, largely confirmed since then, that prostaglandins and other lipid-related mediators are formed and released upon stimulation of a sequential pathway, consisting of activation of phospholipases A2 and subsequently of the metabolisation of the released unsaturated fatty acids, particularly AA [23–26]. More largely, this reinforced the concept that a variety of lipid and lipid-related substances exert injurious or protective effects when released at sufficient concentrations at the proper sites. The initial trigger for the release of these mediators is Ca^{2+}-dependent activation of phospholipase A2, the presently recognized limiting step for prostaglandin biosynthesis. (see [27] for initial evidence).

The identification of RCS with TxA2 [28] enabled this general concept to be adapted to platelet aggregation, and to arterial thrombosis. A membrane signal facilitates the entry and/or translocation of Ca^{2+} to sites containing phospholipase A2, leading to release of AA from available phospholipids or, more precisely, from those situated in the dense tubular system, where cyclooxygenase and in certain cells or fragments, thromboxane synthetase are found as well [29–31].

The arachidonate pathway is considered presently as a second pathway for aggregation [6, 32, 33], mediated by TxA2 formed from the PG endoperoxides. The correlation between inhibition of cyclooxygenase and suppression of platelet aggregation by AA [34], led to an overestimation of the role of the metabolites of AA for aggregation in general, and thus to undue hopes that NSAID would show clinically useful anti-thrombotic properties, because they prevent the synthesis of TxA2. In fact, there is no doubt that inhibition of the synthesis of PG correlates with antagonism of AA-induced aggregation. This can be clearly observed with the use of stereoisomers of anti-inflammatory drugs (Fig. 2). Even though platelet aggregation due to thrombin and to collagen is accompanied by synthesis of TxA2,

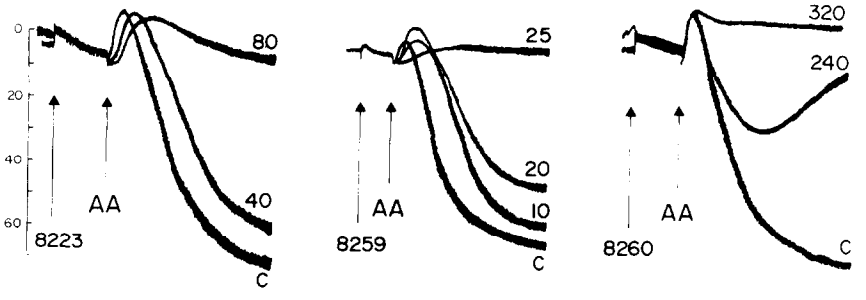

Fig. 2. Stereospecificity of inhibition of arachidonic acid-induced aggregation of rabbit platelets. Superimposed tracings of aggregation induced by 0.1 mM (final concentration) of arachidonic acid (AA), in the presence and in the absence of the indicated concentration (μg/ml) of the anti-inflammatory agent *dl*-6-methoxy-1,2,3,4 tetrahydrocarbazole-2-carboxylic acid (8223 is the code number for the racemate, 8259 for the levo-isomer, which is the active species, and 8260 for the dextro-isomer, which is the inactive species). Large concentrations of the latter (above 300 μg/ml) suppress aggregation by ADP as well, indicating a non-specific effect. 8223 and 8259 are effective anti-inflammatory agents (drugs kindly provided by Organon).

which is inhibited by NSAID, the anti-aggregating activity of these drugs does not extend automatically from AA to other, more relevant, aggregating agents (see below: 'Thrombin-induced Aggregation'). Moreover, the mode of action of the NSAID is not only limited to inhibition of cyclo-oxygenase: aspirin-like drugs (which in fact should not be called any further by this generic and trivial denomination, because of the multiplicity of their effects); they inhibit peroxidase of human platelets [35], and indomethacin has been reported to inhibit human platelet phospholipase A2 [36], when directly applied to a platelet fraction displaying such activity upon a semi-synthetic substrate. However, we failed to confirm this inhibition, when indomethacin was applied to human platelets, with or without drugs removal, before the platelets were stimulated with thrombin, according to refs [30] and [37] (Vargaftig, Fouque and Chignard, in preparation).

It should also be stressed that Tx's are detected in the supernatant of thrombin stimulated platelets only when the concentrations applied are quite above those which are needed to trigger aggregation; for low amounts of thrombin, when aggregation is inhibitable by NSAID, no TxA2 nor TxB2 (Fig. 3) can be found, even though these amounts of thrombin do trigger full aggregation. Even though TxA2 can be released by the outer membrane the important event for Tx-dependent aggregation is its release within the platelet: TxA2 is rather an intracellular mediator than a cell-to-cell messenger.

Finally, if platelet cyclooxygenase is inactivated by aspirin, aggregation induced by threshold amounts of thrombin is inhibited, but it suffices to increase by 2 or 4 fold the amounts of thrombin, to overcome inhibition, and obtain a full platelet reponse even though the underlying formation of TxA2 remains inhibited [6, 9, 32, 33, 38]. Furthermore, the association of an enzymatic system which destroys ADP as soon as it is released from the platelet, with aspirin or with indomethacin, also fails to inhibit aggregation by moderate to high concentrations of thrombin [6, 9]. When the ADP stocks of the platelets are exhausted by submitting the platelets to thrombin, before their resuspension, reactivity to thrombin itself is lost, but the platelets respond to ADP or to AA, provided fibrinogen is added to the suspension [11, 39]. Thrombin-treated platelets aggregate also when stimulated with convulxin, a substance contained in the venom of rattlesnakes [40], even when indomethacin is present (Fig. 1). This all confirms that the first and the second pathways can be suppressed and platelets still maintain their reactivity (see below: 'Convulxin, Another Thromboxane and ADP-independent Platelet Stimulator').

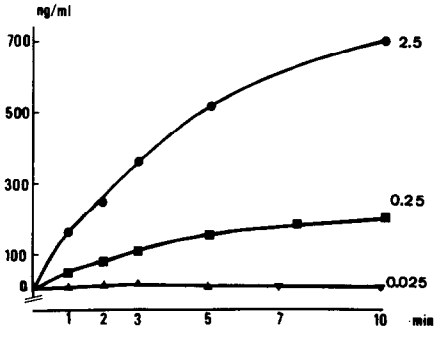

Fig. 3. Release of thromboxane B2 by thrombin-stimulated rabbit platelets. Rabbit platelets suspended in Tyrode's solution were stimulated with thrombin, at concentrations indicated near each curve, in U/ml. Samples were collected at the indicated intervals (min, horizontal scale), and thromboxane B2 was determined by radioimmune assay (ng/ml, vertical scale). Thromboxane was undetectable when the platelets were stimulated with 0.025 U/ml of thrombin, even though aggregation was immediate.

THE THIRD PATHWAY OF AGGREGATION

We recently obtained evidence that a phospholipid, namely 1-*O*-alkyl-2-*O*-acetyl-2*sn*-glyceryl-3-phosphorylcholine (platelet-activating factor, PAF-acether) [41–44] may account for the third pathway of aggregation [45–49]. PAF-acether is known to be released from various cells types upon immune and non-immune challenge [50], and is one of the most powerful aggregating substances so far described, since concentrations as low as 1 nM trigger the aggregation of washed rabbit platelets (Fig. 4). In the presence of plasma, its activity is markedly reduced, but since PAF-acether appears to be released within the platelet (as is TxA2, see above), the reduction of its activity by plasma should not be retained as evidence against its role. As seen in Fig. 5, guinea-pig platelets are more sensitive to PAF-acether than rabbit platelets, whereas human platelets are even less. Aggregation by PAF-acether is not affected by the ADP scavenging system creatine phosphate/creatine phosphokinase, nor by the inhibitors of cyclooxygenase [45, 50]. Moreover, thrombin-degranulated platelets also aggregate when stimulated with PAF-acether [45]. Aggregation of guinea-pig [51] and of rabbit [45] platelets appears not to be accompanied by formation of TxA2, but there is evidence for formation of TxA2 in one case [52]. Both ADP and ATP are released by PAF-acether. Since prostacyclin and bivalent metal chelating-agents suppress aggregation by PAF-acether [51], and since the cytoplasmatic marker lactic

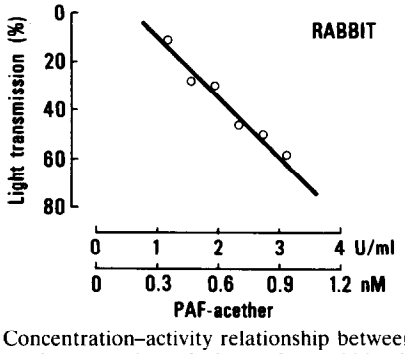

Fig. 4. Concentration–activity relationship between PAF-acether and aggregation of plasma-free rabbit platelets. The light transmission across the platelet suspension increases with increasing amounts of PAF-acether added. The figure also provides the correspondence between the previously used arbitrary units of aggregation, and actual concentrations of synthetic PAF-acether (kindly provided by Dr. J.-F. Godfroid, University of Paris VI).

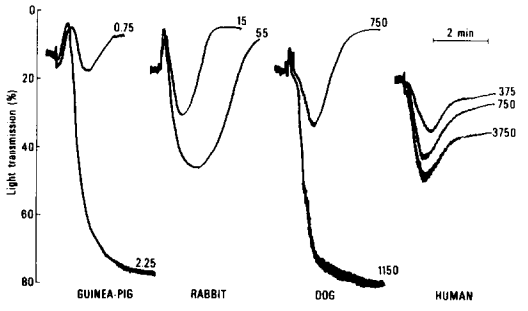

Fig. 5. Species-dependent responsiveness of platelets in plasma to PAF-acether. Superimposed tracings of platelet aggregation by synthetic PAF-acether (nM) for the indicated species.

dehydrogenase is not released, one may conclude that PAF-acether induces authentic aggregation, liable to inhibition by increasing the adenylate cyclase activity of the platelet.

Aspirin does not inhibit the platelet aggregation by PAF-acether [45, 46, 51], but when administered together with anti-histaminic and anti-serotonin agents, which alone fail to interfere with the effects of PAF-acether, and the latter's bronchoconstrictor effects in the guinea-pig are suppressed (to be published). Furthermore, a similar synergism is present for indomethacin, and for the non-cyclooxygenase inhibitor salicylic acid, when administered together with the antiamine substances. Finally, sulfinpyrazone, presently used as a anti-thrombotic agent, alone inhibits aggregation by PAF-acether *in vitro*, and bronchoconstriction *in vivo*, whereas the accompanying thrombocytopenia is unaffected (Vargaftig, Lefort, Chignard, Wal and Benveniste, in preparation).

The mode of release of PAF-acether from the platelets is presently unknown. The most active releasing agent is the Ca^{2+} ionophore A23187, but large amounts, enough to account for aggregation, are released from rabbit platelets by collagen and by thrombin. In contrast, ADP and AA fail to trigger release of PAF-acether [48, 49], giving weight for our hypothesis for a specific role of this mediator. Release of PAF-acether differs from that of all other mediators, in that it is a phospholipid, a piece of the membrane. In this respect, it might be argued that release of PAF-acether from platelets is indicative, rather than a cause, of membrane alterations accompanying aggregation. Failure of ADP and of AA to imitate collagen or thrombin with respect to PAF-acether argues against this possibility. When thrombin or collagen activate platelets, phospholipase A2 is activated as well, and this accounts for the release of the PG precursor AA from the phospholipids. As stated below (see 'Role of Phospholipase A2'), three inhibitors of phospholipase A2 (bromophenacyl bromide, a compound known as CB 874 and mepacrine) block platelet aggregation by thrombin, and the accompanying release of AA and of TxA2. The phospholipase A2 inhibitors also suppress aggregation by, and release of, PAF-acether. The specificity of each individual inhibitor of phospholipase A2 is questionable, and their activity may result in profound membrane disturbances (see below: 'Membrane Phospholipids and Phospholipase A2'). Inhibition of the release of PAF-acether by these reagents may in fact be due to these membrane effects, and not really to enzyme inhibition. This is furthermore supported by the fact that the so-called membrane-active drugs are very active in suppressing the direct effects of PAF-acether [45]. The latter would thus be a very particular reagent, in that both its release from the platelets, as well as its effect on the platelet, result from membrane disturbances controllable by inhibitors of phospholipase A2.

Convulxin, another thromboxane and ADP-independent platelet stimulator

Convulxin(Cx) is a glycoprotein present in the venom of snakes belonging to the genus *Crotalus* (South American rattlesnakes). It is active in releasing serotonin and ATP from rabbit platelets, and it triggers aggregation, accompanied by the release reaction, when applied to platelets of rabbits, guinea-pigs, mice, humans and dogs ([40, 53]; Marlas and Joseph, in preparation; Fig. 1). Platelet activation by threshold amounts of Cx, as measured by aggregation and by the release of ATP, is reduced by scavengers of ADP and by aspirin, but it suffices to increase by 2 or 4 times the concentration of Cx used, to overcome inhibition (Fig. 1). It thus appears that Cx induces ADP and TxA2-independent aggregation, involving a third mechanism. This concept is confirmed by our unpublished results, showing that rabbit platelets stimulated by thrombin, which do not respond to a second stimulus with thrombin itself, and are depleted from their granular ADP, are still aggregated by Cx. Furthermore, when the thrombinized platelets are recovered, and further stimulated with Cx, TxA2 is released, and phospholipase A2 is activated, as indicated by loss of labelled arachidonate from phospholipids. Thus, even though platelets are desensitized to thrombin, they remain responsive to other stimulants of phospholipase A2.

Evidence for a pivotal role of phospholipase A2

As stated above, phospholipase A2 appears to be the limiting step for the synthesis of TxA2 [27, 30]. Activation of this enzyme should nevertheless lead to release of other substances as well [33], which appear to be involved with the ADP and TxA2-independent platelet aggregation, and are discussed in the following paragraphs.

Platelets contain phospholipases and in their pioneering study Smith and Willis [21] demonstrated the presence of a poorly characterized phospholipase in the supernatant of thrombin-stimulated platelets. The concept that prostaglandins are released from endogenous precursors only after the activation of an acylhydrolase such as phospholipase A2 arose from the early work of Vogt [27], and was later adapted for platelets. The rapid development of a methodology for the study of enzyme activation in whole platelets, allowed for a rapid progress in the identification of the phospholipid pools which provide arachidonate for the cyclooxygenase complex, i.e. phosphatidylcholine and/or phosphatidylinositol. There is no doubt presently that TxA2 and TxB2, released from platelets stimulated with thrombin,

with collagen and with the calcium ionophore A23187, are formed from the AA hydrolized from those phospholipids. Inhibition of the cyclooxygenase-dependent pathway by aspirin is followed by a large rise in the yield of aliphatic derivatives of arachidonate, which come from the same phospholipids, giving further weight to the critical role of phospholipase A2. Platelet phospholipase A2 can be inhibited with mepacrine [54, 55] found previously to inhibit the release of thromboxane A2 from guinea-pig lungs injected with bradykinin [23]. Aggregation by collagen is also inhibited by mepacrine but the concentrations required are close to those which block primary aggregation by ADP as well. The first irreversible inhibitor of phospholipase A2 to be tested on the platelets was bromophenacyl bromide [33] previously shown to inhibit the phospholipase A2 activity of soluble enzymes [56]. It blocks aggregation of washed rabbit and human platelets induced by thrombin and by the calcium ionophore A23187. Use of bromophenacyl bromide requires caution, since it is a potent membrane-active agent, and in proper amounts and time of contact with the platelets, it reduces the yield of thromboxane A2 formed directly from added AA [37]. It shows nevertheless a preferential effect over phospholipase A2 when incubated for short intervals only with the platelets [37]. Compound CB 874 also inhibits phospholipase A2 and shows anti-inflammatory properties in the rat, as well as anti-platelet aggregating activity [57]. CB 874 is relatively specific for phospholipase A2 as compared to cyclooxygenase, but is probably toxic for the platelet membrane, since it induces the release of lactic dehydrogenase (Vargaftig, Fouque and Chignard, unpublished).

Corticosteroids inhibit the release of the metabolites of AA from tissues other than platelets. This has been attributed to three possible mechanisms: a membrane protective effect, similar to that described for the inhibition of the release of lysosomal enzymes from leukocytes, interference with the availability of AA (a sort of pseudo-inhibition of phospholipase A2 or of its activation), and finally, a classical receptor-mediated effect, with synthesis of polypeptide with anti-phospholipase A2 properties [55, 58–61]. Failure of corticosteroids to inhibit phospholipase A2 activity of blood platelets might be due to the absence of nucleus, and consequently, of the ability to synthesize the hypothetical inhibitory peptide. Nevertheless, when chicken thrombocytes, which are nucleated, were used in place of anucleated platelets, glucocorticoids were still inactive against collagen-induced aggregation and formation of TxB2. Under those conditions, the aggregating effects of AA are observed, and are inhibited by NSAID. Thus, glucocorticoids do not inhibit the activity of phospholipase A2 in blood platelets and thrombocytes, when applied *in vitro* (F. Russo-Marie and C. Ody, unpublished). *In vivo* experiments, where megakaryocytes might have been affected, were not reported.

Platelet membrane phospholipids and phospholipase A2

Platelet membrane phospholipids may participate in the modulation of the platelet release reaction [62]. Furthermore, recent works on mast cells [63], on chromaffin granules of the adrenal gland [64], on rabbit leukocytes [65], murine T lymphocytes [66] and rat reticulocyte ghosts [67] show that cell activation causes a transient increase in the methylation of phosphatidylethanolamine to phosphatidyl choline by two very different methyltransferases. Accumulation of intermediate phosphatidyl monomethylethanolamine increases the membrane fluidity of T lymphocytes [66], leading presumably to increased Ca^{2+} influx and activation of phospholipases. Mepacrine and hydrocortisone inhibit the degradation of methylated phospholipids of rabbit leukocytes stimulated by chemoattractant peptides (65], again suggesting a close association between metabolism of methylated phospholipids and chemotaxis.

Evidence against the pivotal role of phospholipase A2

Recent results casted some doubt on the role of phospholipase A2 for platelet aggregation. This includes our results with carrageenan and new concepts on the early activation of phopholipase C.

Carrageenan

Aggregation of washed human and rabbit platelets by the polysaccharide carrageenan is reduced by NSAID [33, 68–77], but this partial inhibition is not seen when the inhibitors of cyclooxygenase are removed, even though inhibition of formation of prostaglandins and of TxA2 or TxB2 remains. Moreover, salicylic acid inhibits aggregation by carrageenan under conditions similar to those of aspirin. Since salicylic acid is not an inhibitor of cyclooxygenase, we concluded that its inhibiting activity towards aggregation by carrageenan resulted from its physical presence in the medium, possibly because, at the concentrations required to affect carrageenan, the NSAID interfere with Ca^{2+} movements through the various platelet membranes. In contrast, application of bromophenacyl bromide or of CB 874 to carrageenan-stimulated platelets, suppresses altogether aggregation and the accompanying release reaction [70]. Nevertheless, only the rabbit platelets form thromboxane when stimulated by carrageenan, whereas human platelets fail to do so. This means that the phospholipase inhibitors also interfere with aggregation when phospholipase is not involved. In agreement, carrageenan fails to trigger the release of labelled arachidonate, from human platelets, under conditions where thrombin is active. The examination of the phospholipid subspecies one by one, with thin-layer chromatography, failed to demonstrate a specific site in which carrageenan would act when human platelets are used. It might be argued that the methods used for demonstrating activation of phospholipase A2 are not sufficiently sharp to show activation on a critical site, overshadowed by the bulk of the full platelet activity. This is still open to controversy.

The phospholipase C hypothesis

Release of arachidonate by a pathway other than activation of phospholipase A2 has recently been described. It consists of the activation of a phos-

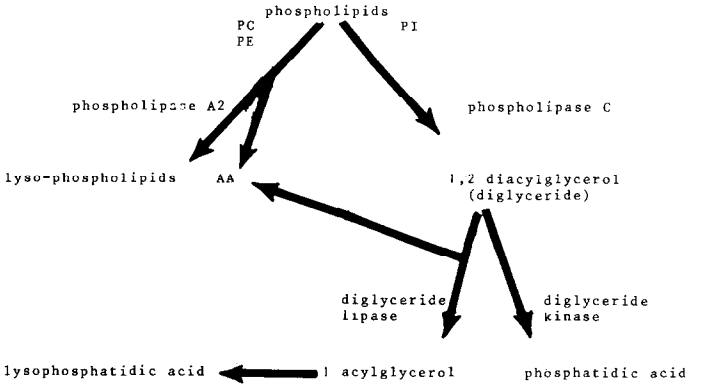

Fig. 6. The two enzymatic pathways triggering arachidonic acid release.

phatidylinositol phosphodiesterase (phospholipase C), with formation of diglycerides [72–79]. The presence of phospholipase C in platelets was indicated by the stimulation of the production of phosphatidic acid in platelets before the release of AA [73]. The diglycerides formed from phosphatidylinositol upon stimulation of phospholipase C are the immediate precursors of phosphatidic acid, formed upon stimulation of a diglyceride kinase [73, 74–76]. Alternatively, under certain conditions, such as platelet stimulation with the ionophore A23187, a diglyceride lipase is stimulated, and 1-acylglycerol and AA are thus formed [75, 78]. Arachidonate comes thus from at least two different pathways: phospholipase A2, acting preferentially on phosphatidylethanolamine and phosphatidylcholine, and phospholipase C, acting on phosphatidylinositol (Fig. 6; see also ref. [80]). Both enzymes are Ca^{2+}-dependent and under the control of the cyclic AMP system. The phospholipase C pathway provides high amounts of phosphatidic acid [72, 73, 76–80], a potential trigger for aggregation, which releases Ca^{2+} from a platelet membrane fraction, and is thus a Ca^{2+} ionophore [81]. Phosphatidic acid does not cross the platelet membrane, and thus cannot trigger aggregation when added to a platelet suspension.

Exogenous phospholipases C from different sources have been tested on platelets. *Bacillus cereus* phospholipase C, which does not aggregate platelets, degrades glycerophospholipids other than phosphatidylinositol whereas *Clostridium perfringens* phospholipase C, which aggregates platelets, hydrolyses all phospholipids including sphingomyelin [72, 78, 79, 82]. The difference in platelet activity of the two phospholipases is probably due to the hydrolysis of sphingomyelin, rather than of phosphatidylinositol, since a combination of a specific sphingomyelinase C from *Staphylococcus aureus* with the phospholipase C from *B. cereus* triggers aggregation accompanied by a release reaction and by the formation of TxA2 [79]. At the highest concentrations of *C. perfringens* phospholipase C platelet lysis is rapid (Fig. 7). In contrast, for low concentrations of this phospholipase C, lysis appears slowly and probably does not account for the release of ATP nor for the increase in light transmission, since the kinetics of the different events is not coincidental (Fig. 7). Synthesis of phosphatidic acid is also increased during platelet aggregation by *C. perfringens* phospholipase C [78]. Under those conditions, it is possible that phosphatidic acid formed within the platelet is responsible for aggregation, since the amounts of TxA2 formed are low (around fifteen times below those found after stimulation with thrombin), and furthermore aspirin fails to block aggregation by the phospholipase C, in contrast to effectiveness against phospholipase A2 [83]. Inhibitors of the latter, such as bromophenacyl bromide or CB 874, fail to interfere with platelet lysis due to *C. perfringens* phospholipase C, but inhibit the underlying aggregation, particularly when due to threshold concentrations of the enzyme. This does not prove that phospholipase C triggers aggregation via phospholipase A2, since the inhibitors display unclear membrane effects, as stated above (unpublished results).

CONCLUDING REMARKS

The different mechanisms of platelet aggregation (at least for rabbit) can be schematically represented by an hydraulic system in which the different aggregating agents can push specific pistons (Fig. 8). Thus the calcium ionophore, thrombin and collagen put pressure on phospholipase A2 (PLA2) which can be suppressed by closure of a floodgate triggered by bromophenacyl bromide (BPB) or dibutyryl AMP cyclic (dbAMPc). It can be further speculated that the fluid is formed by calcium ions. Pressure is then transmitted by means of two other fluids which are arachidonic acid (AA) and PAF-acether (PAF). This phospholipase A2 (PLA2) step can be short-cut by adding directly AA or PAF-acether to the system. After its passage through the cyclooxygenase (CO) enzyme system, AA originates thromboxane A2 (TxA2), which triggers aggregation directly and indirectly by transmission of the pressure to ADP. The same mechanism is applicable to PAF-acether, which acts directly and indirectly via ADP.

This scheme is clearly an oversimplification of the living system. Thus it does not take into account the

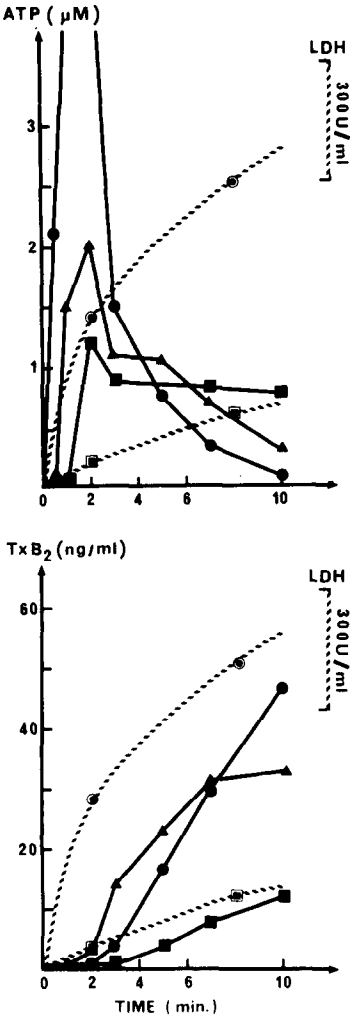

Fig. 7. Comparison between the release of ATP and the formation of thromboxane B2 by human platelets challenged with phospholipase C. A suspension of plasma-free washed human platelets was challenged with *C. perfringens* phospholipase C, which had been preincubated with cholesterol, to inactivate the contaminant θ toxin. The release of ATP is indicated in the upper panel, and the formation of TxB2 in the lower panel, for three concentrations of phospholipase C (0.3 U/ml, circles; 0.1 U/ml, triangles; 0.03 U/ml, squares). The lytic activity of the lowest and of the highest concentration of phospholipase C was monitored by measuring the lactic dehydrogenase activity in the platelet supernatant (dotted lines). Platelet lysis cannot account for aggregation (not shown) and release of ATP induced by the lowest concentration of phospholipase C. In contrast, lysis induced by the highest concentration of the enzyme is probably responsible for the increase in light transmission observed as being aggregation. Formation of TxB2 appears to be due to platelet lysis, at both concentrations of phospholipase, particularly since aggregation induced by thrombin is accompanied by a markedly higher amount of TxB2 (see Fig. 3).

product of the enzymatic activity of the phospholipase C such as phosphatidic acid and lysophosphatidic acid which can play a role in aggregation. Nevertheless it shows that when cyclo-oxygenase is inhibited and ADP is scavenged the third mechanism still operates. Only phospholipase A2 inhibitors can

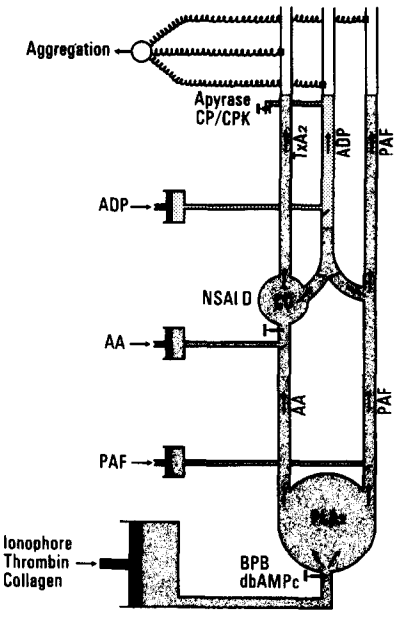

Fig. 8. Hypothetical scheme demonstrating the implication of the three different pathways leading to aggregation of rabbit platelets. (See commentary in text.)

block the three pathways altogether. Moreover, this scheme considers collagen as a trigger of the third pathway, which has not always been the opinion of all authors [6, 9, 10]. It appears presently that indeed collagen-induced aggregation can proceed when the first and the second pathways are suppressed, and that contradictory results were due to the use of insufficient amounts of collagen, together with a competitive ADP-scavenger such as the creatine phosphate/creatine phosphokinase system [84].

The PAF-acether hypothesis for the third pathway of platelet aggregation may be validated or eliminated in the near future. Many aspects of platelet activation remain obscure, and were not reviewed here, including the role of calmodulin, of Ca^{2+} as a multipotent mediator, or of glycoproteins, platelet fibrinogen, and the products of the lipooxygenase pathway. A better understanding of the third pathway of aggregation, in connection with the other platelet properties, should allow us to build up better anti-thrombotic drugs, and to better understand the mode of action of the present ones.

Acknowledgements—Original work mentioned in this paper was done in collaboration with Mrs. F. Fouque, Ms. C. Dumarey, and MM. J.-P. le Couedic and J. Lefort, who are gratefully acknowledged.

REFERENCES

1. N. L. Baenziger, G. N. Brodie and P. W. Majerus, *Proc. natn. Acad. Sci. U.S.A.* **68**, 240 (1971).
2. D. M. Tollefsen, J. R. Feagler and P. W. Majerus, *J. biol. Chem.* **249**, 2646 (1974).
3. T. Okumura, M. Hasitz and G. A. Jamieson, *J. biol. Chem.* **253**, 3435 (1978).
4. S. W. Tam and T. C. Detwiler, *Biochim. biophys. Acta* **543**, 194 (1978).
5. J. P. Allain, H. A. Cooper, R. H. Wagner and K. M. Brinkhous, *J. lab. clin. Med.* **85**, 318 (1975).

6. R. L. Kinlough-Rathbone, M. A. Packham, H. J. Reimers, J. P. Cazenave and J. F. Mustard, *J. lab. clin. Med.* **90**, 707 (1977).
7. H. Holmsen and H. J. Weiss, *Ann. Rev. Med.* **30**, 119 (1979).
8. R. J. Haslam, *Nature* **202**, 765 (1964).
9. M. A. Packham, M. A. Cuccione, J. P. Greenberg, R. L. Kinlough-Rathbone and J. F. Mustard, *Blood* **50**, 915 (1977).
10. I. F. Charo, R. D. Feinman and T. C. Detwiler, *J. clin. Invest.* **60**, 866 (1977).
11. R. L. Kinlough-Rathbone, A. Chahil, M. A. Packham, H. J. Reimers and J. F. Mustard, *Thromb. Res.* 7, 435 (1975).
12. R. L. Kinlough-Rathbone, H. J. Reimers, J. F. Mustard and M. A. Packham, *Science* **192**, 1011 (1976).
13. I. F. Charo, R. D. Feinman, T. C. Detwiller, J. B. Smith, C. M. Ingerman and M. J. Silver, *Nature* **269**, 66 (1977).
14. B. B. Vargaftig, *Experientia* **22**, 182 (1966).
15. P. J. Piper and J. R. Vane, *Nature* **223**, 29 (1969).
16. B. B. Vargaftig, E. P. Miranda and B. Lacoume, *Nature* **222**, 883 (1969).
17. B. B. Vargaftig and N. Dao, *Pharmac. Res. Commun.* **2**, 149 (1970).
18. B. B. Vargaftig and N. Dao, *Pharmacology* **6**, 99 (1971).
19. J. R. Vane, *Nature (New Biol.)* **231**, 232 (1971).
20. S. H. Ferreira, S. Moncada and J. R. Vane, *Nature (New Biol.)* **231**, 237 (1971).
21. J. B. Smith and A. L. Willis, *Nature (New Biol.)* **231**, 235 (1971).
22. S. H. Ferreira and B. B. Vargaftig, *Br. J. Pharmac.* **50**, 543 (1974).
23. B. B. Vargaftig and N. Dao Hai, *J. Pharm. Pharmac.* **24**, 159 (1972).
24. B. B. Vargaftig, in *The Prostaglandins* (Ed. P. Ramwell), Vol. 2, p. 205. Plenum Press, New York (1974).
25. B. B. Vargaftig and M. Chignard, *Agents Act.* **5**, 137 (1975).
26. M. Chignard and B. B. Vargaftig, in *Actualités de Physiologie Pathologique* (Eds J. L. Parrot and F. Ruff), p. 127. Expansion Scientifique, Paris (1979).
27. W. Vogt and B. Distelkotter in *Prostaglandins*, Proceedings of the Second Nobel Symposium Stockholm 1966 (Eds. S. Bergstrom and B. Samuelsson) p. 237. Interscience, New York (1967).
28. M. Hamberg, J. Svensson and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **72**, 2994 (1975).
29. S. Rittenhouse-Simmons, F. A. Russell and D. Deykin, *Biochim. biophys. Acta* **488**, 370 (1977).
30. T. K. Bills, J. B. Smith and M. J. Silver, *Biochim. biophys. Acta* **424**, 303 (1976).
31. G. J. Blackwell, W. G. Duncombe, R. J. Flower, M. F. Parsons and J. R. Vane, *Br. J. Pharmac.* **59**, 353 (1977).
32. E. G. Lapetina, K. A. Chandrabose and P. Cuatrecasas, *Proc. natn. Acad. Sci. U.S.A.* **75**, 818 (1978).
33. B. B. Vargaftig, *J. Pharm. Pharmac.* **29**, 222 (1977).
34. B. B. Vargaftig and P. Zirinis, *Nature (New Biol.)* **244**, 114 (1973).
35. M. I. Siegel, R. T. McConnell and P. Cuatrecasas, *Proc. natn. Acad. Sci. U.S.A.* **76**, 3774 (1979).
36. R. L. Jesse and R. C. Franson, *Biochim. biophys. Acta* **575**, 467 (1979).
37. B. B. Vargaftig, F. Fouque and M. Chignard, *Thromb. Res.* **17**, 91 (1980).
38. B. B. Vargaftig, M. Chignard and J. Lefort, *in Advances in Pharmacology and Therapeutics* (Ed. B. B. Vargaftig) Vol. 4, p. 27. Pergamon Press, Oxford (1978).
39. H. J. Reimers, M. A. Packham, R. L. Kinlough-Rathbone and J. F. Mustard, *Br. J. Haemat.* **25**, 675 (1973).
40. B. B. Vargaftig, J. Prado-Franceschi, M. Chignard, J. Lefort and G. Marlas, *Eur. J. Pharmac*, in press.
41. J. Benveniste, P. M. Henson and C. G. Cochrane, *J. exp. Med.* **136**, 1356 (1972).
42. C. A. Demopoulos, R. N. Pinckard and D. J. Hanahan, *J. biol. Chem.* **254**, 9355 (1979).
43. J. Benveniste, M. Tence, P. Varenne, J. Bidault, C. Boullet and J. Polonsky, *C. R. Acad. Sci. Paris (D)* **189**, 1037 (1979).
44. J. Benveniste and B. B. Vargaftig, *Proceedings of the 12th. International Course on Transplantation and Clinical Immunology*, Excerpta Medica (in press).
45. J. P. Cazenave, J. Benveniste and J. F. Mustard, *Lab. Invest.* **41**, 275 (1979).
46. M. Chignard, M. Tence, J. P. Le Couedic, B. B. Vargaftig and J. Benveniste, *Fedn. Proc.* **38**, 1342 (1979).
47. M. Chignard, J. P. Le Couedic, M. Tence, J. Benveniste and B. B. Vargaftig, *J. Pharmac. (Paris)* **10**, 259 (1979).
48. M. Chignard, J. P. Le Couedic, M. Tence, B. B. Vargaftig and J. Benveniste, *Nature* **179**, 799 (1979).
49. M. Chignard, J. P. Le Couedic, B. B. Vargaftig and J. Benveniste, *Br. J. Haemat* **46**, 455 (1980).
50. B. B. Vargaftig, M. Chignard, J. Lefort and J. Benveniste, *Agents Act.* (in press).
51. B. B. Vargaftig, J. Lefort, M. Chignard and J. Benveniste, *Eur. J. Pharmac* **65**, 185 (1980).
52. J. O. Shaw, M. P. Printz, K. Hirabayashi and P. M. Henson, *J. Immunol.* **121**, 1939 (1978).
53. F. Markwardt, W. Barther, E. Glusa and A. Hoffman, *Archs exp. Path. Pharmak.* **252**, 297 (1966).
54. E. Vallee and J. F. Delahayes, *Eur. J. Rheumatol. Inflam.* **1**, 3 (1978).
55. G. J. Blackwell, R. J. Flower, F. P. Nijkamp and J. R. Vane, *Br. J. Pharmac.* **62**, 79 (1978).
56. J. J. Volwerk, W. A. Pieterson and G. H. deHaas, *Biochemistry* **13**, 1446 (1974).
57. E. Vallee, J. Gougat, J. Navarro and J. F. Delahayes, *J. Pharm. Pharmac.* **31**, 588 (1979).
58. M. W. Greaves and W. McDonald-Gibson, *Br. Med. J.* **2**, 83 (1972).
59. G. P. Lewis and P. J. Piper, *Nature* **254**, 308 (1975).
60. R. J. Gryglewski, B. Panczenko, R. Korbut, L. Grodzinska and A. Ocetkiewicz, *Prostaglandins* **10**, 343 (1975).
61. F. Russo-Marie, M. Paing and D. Duval, *J. biol. Chem.* **254**, 8498 (1979).
62. P. K. Schick and B. P. Yu, *J. clin. Invest.* **54**, 1032 (1974).
63. T. Ishizaka, F. Hirata, K. Ishizaka and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **77**, 1903 (1980).
64. F. Hirata, O. H. Viveros, E. J. Diliberto Jr. and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **75**, 1718 (1978).
65. F. Hirata, B. A. Corcoran, K. Venkatasubramanian, E. Schiffmann and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2640 (1979).
66. F. Hirata, S. Toyoshima, J. Axelrod and M. J. Waxdal, *Proc. natn. Acad. Sci. U.S.A.* **77**, 862 (1980).
67. F. Hirata, W. J. Strittmatter and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **76**, 368 (1979).
68. R. M. McMillan, D. E. MacIntyre and J. L. Gordon, *J. Pharm. Pharmac.* **31**, 148 (1979).
69. G. Kindness, W. F. Long, F. B. Williamson and J. Boyd, *Thromb. Res.* **15**, 3 (1979).
70. B. B. Vargaftig, F. Fouque, M. Chignard and C. Dumarey, *J. Pharm. Pharmac.* **32**, 740 (1980).
71. B. B. Vargaftig, in *Recent Developments in the Pharmacology of Inflammatory Mediators* (Ed. I. L. Bonta), *Agents and Actions* Suppl. 2, p. 9. Birkhaüser Verlag Basel (1977).
72. G. Mauco, H. Chap and L. Douste-Blazy, *Febs. Lett.* **100**, 367 (1979).

73. E. G. Lapetina and P. Cuatrecasas, *Biochim. biophys. Acta* **573**, 394 (1979).
74. S. Rittenhouse-Simmons, *J. Clin. Invest.* **63**, 580 (1979).
75. R. L. Bell, D. A. Kennerly, N. Stanford and P. W. Majerus, *Proc. natn. Acad. Sci. U.S.A.* **76**, 3238 (1979).
76. M. M. Billah, E. G. Lapetina and P. Cuatrecasas, *Biochem. biophys. Res. Commun.* **90**, 92 (1979).
77. R. L. Bell and P. W. Majerus, *J. biol. Chem.* **225**, 1790 (1980).
78. G. Mauco, H. Chap, M. F. Simon and L. Douste-Blazy, *Biochimie* **60**, 653 (1978).
79. H. Chap, B. Perret, G. Mauco, M. F. Simon and L. Douste-Blazy, *Agents Act.* **9**, 400 (1979).
80. J. V. Lloyd and J. F. Mustard, *Br. J. Haemat.* **26**, 243 (1974).
81. J. M. Gerrard, A. M. Butler, D. A. Peterson and J. G. White, *Prost. Med.* **1**, 387 (1978).
82. H. Chap and L. Douste-Blazy, *Eur. J. Biochem.* **48**, 351 (1974).
83. D. Heinrich and S. Beckmann, *Throm. Haemostas.* **42**, 246, abst 0581 (1979).
84. R. L. Kinlough-Rathbone, J. P. Cazenave, M. A. Packham and J. F. Mustard, *Lab. Invest.* **42**, 28 (1980).