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PROSTAGLANDIN ENDOPEROXIDES, THROMBOXANE A_2 AND ADENOSINE DIPHOSPHATE IN COLLAGEN-INDUCED AGGREGATION OF RABBIT PLATELETS

G. P. LEWIS & I. S. WATTS

Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons, Lincoln's Inn Fields, London WC2A 3PN

1 A bioassay technique is described for simultaneously monitoring rabbit platelet aggregation with measurement of thromboxane A_2 (TxA_2) and prostaglandins released in response to collagen or arachidonic acid (AA).

2 Five imidazole derivatives were examined as inhibitors of thromboxane synthetase and compared with the effect of the cyclo-oxygenase inhibitor indomethacin; 1-(7-carboxyheptyl) imidazole was identified as the most potent and selective inhibitor of thromboxane synthetase and was used with indomethacin to investigate the relative contribution of the prostaglandin endoperoxides prostaglandin G_2 (PGG_2)/PGH_2 and TxA_2 in mediating platelet aggregation induced by collagen or AA.

3 Platelet aggregation induced by a low concentration of collagen was abolished by indomethacin and carboxyheptylimidazole whilst in response to a high concentration or collagen only partial inhibition of aggregation occurred.

4 The contribution of adenosine diphosphate (ADP) released from platelets during collagen or AA-induced aggregation was examined using the substrate/enzyme complex creatine phosphate/creatine phosphokinase (CP/CPK). The CP/CPK complex abolished aggregation induced by a low dose of collagen whilst aggregation to a high dose of collagen was only partially inhibited.

5 Aggregation induced by a high dose of collagen was abolished by a combination of CP/CPK with indomethacin or carboxyheptylimidazole.

6 AA-induced aggregation was abolished by indomethacin. Carboxyheptylimidazole abolished aggregation induced by a low dose of AA but inhibition was surmounted with increasing concentrations of AA in the absence of TxA_2 formation.

7 PGH_2-induced aggregation was unaffected by indomethacin and only partially inhibited by carboxyheptylimidazole. AA or PGH_2-induced platelet aggregation was unaffected by CP/CPK.

8 In conclusion, aggregation of rabbit platelets induced by a low concentration of collagen was dependent on synergism between TxA_2 and ADP whilst at high concentrations of collagen, sufficient TxA_2 and ADP were released to induce aggregation independently of each other.

9 The small amounts of prostaglandin endoperoxides produced from endogenous arachidonate have apparently no direct pro-aggregatory role. However, the relatively large amount which can be produced by a high concentration of exogenous AA when TxA_2 formation is prevented can cause aggregation of rabbit platelets.

Introduction

Early studies of collagen-induced platelet aggregation showed that the aggregation was accompanied by the release of adenosine diphosphate (ADP) from platelet granules (Hovig, 1963). The important contribution of ADP to collagen-induced aggregation is still recognized (Kinlough-Rathbone, Packham & Mustard, 1977a; Geratz, Tidwell, Brinkhous, Mohammed, Dann & Loewe, 1978), although its essential role in the mechanism of aggregation has been questioned (Nunn, 1979).

In addition to the role of ADP, it is evident that collagen and other release-inducing agents give rise to the release of arachidonic acid from the phospholipid membrane of platelets. This is metabolized by cyclo-oxygenase to the unstable prostaglandin endoperoxides, prostaglandin G_2 (PGG_2) and PGH_2

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which in turn are converted by thromboxane synthetase to thromboxane A_2 (TxA_2). The endoperoxides PGG_2, PGH_2 and TxA_2 are platelet-aggregating and release-inducing agents in their own right (Vargaftig & Zirinis, 1973; Smith, Ingerman, Kocsis & Silver, 1974; Malmsten, Hamberg, Svensson & Samuelsson, 1975; Hamberg, Svensson & Samuelsson, 1975), although they have also been shown to induce aggregation of platelets independently of granular secretion (Charo, Feinman, Detwiler, Smith, Ingerman & Silver, 1977).

The significant role of the cyclo-oxygenase pathway in collagen-induced aggregation is supported by evidence of partial or complete inhibition of aggregation by the non-steroidal anti-inflammatory drugs, the extent of inhibition being dependent on the concentration of collagen used (Zucker & Peterson, 1970; Silver, Hernandovich, Ingerman, Kocsis & Smith, 1974; Kinlough-Rathbone, Packham, Reimers, Cazenave & Mustard, 1977b).

In the experiments reported here, we have modified a previously described bioassay procedure (Ladd & Lewis, 1980) to enable simultaneous monitoring of collagen-induced platelet aggregation with TxA_2 and prostaglandin release. We have used inhibitors of platelet cyclo-oxygenase and TxA_2 synthetase, alone and in combination with the substrate/enzyme complex creatine phosphate (CP) and creatine phosphokinase (CPK), which catalyses the conversion of ADP released from platelets to adenosine triphosphate (ATP) (Haslam, 1967), to investigate the mechanism(s) of collagen-induced platelet aggregation. In particular, we have attempted to determine the relative importance and interrelationship of ADP release and the formation of cyclo-oxygenase products.

The mechanism by which arachidonic acid induced platelet aggregation was also investigated, with the aim of clarifying the relative contribution of PGG_2 (PGH_2) and TxA_2 to platelet aggregation where clear agreement has yet to be reached (Needleman, Minkes & Raz, 1976; Raz, Minkes & Needleman, 1977; Fitzpatrick & Gorman, 1977; 1978; Best, Holland, Jones & Russell, 1980).

Methods

Preparation of platelet-rich plasma (PRP)

New Zealand white male rabbits (3 to 3.5 kg) were anaesthetized with pentobarbitone sodium (40 mg/kg) by marginal ear vein injection. Blood from a polyethylene cannula inserted in the common carotid artery was mixed with one tenth its volume of 3.8% w/v trisodium citrate solution and PRP prepared by whole blood centrifugation at 200 *g* for 20 min at room temperature. Platelet-poor plasma (PPP) was obtained from the blood remaining after removal of PRP, by further centrifugation at 4000 *g* for 10 min. The platelet count was adjusted to 5×10^8 per ml with autologous PPP and the suspension of platelets stored at room temperature in closed polycarbonate tubes for the duration of the experiment.

Collagen-induced aggregation

Platelet aggregation was monitored by the turbidometric method of Born (1962). PRP (1 ml) was preincubated at 37°C for 2 min in a siliconized glass cuvette before incubation with the inhibitor(s) or corresponding vehicle (10–50 μl) for a 3 min period.

In the original method of Ladd & Lewis (1980), an aliquot of PRP was passed over a collagen strip from the Achilles tendon of a rabbit, to initiate the activation of platelets. In the experiments described in this paper, the collagen strip was replaced by solubilized collagen fibrils (Horm) 5 to 50 μg, which were added to the cuvette of PRP following pretreatment with the inhibitor(s) or corresponding vehicle. Arachidonic acid (AA) was used as the sodium salt.

Assay of thromboxane A_2 and prostaglandins

At intervals following addition of collagen or AA, a 200 μl aliquot was removed from the cuvette and bioassayed for TxA_2 and prostaglandin-like activity on a tissue cascade composed of a spiral strip of rabbit aorta (RA) and a rat stomach fundus strip (RSS). In some experiments a rabbit coeliac artery (RCA) was used to distinguish between TxA_2 and prostaglandin endoperoxides (Bunting, Moncada & Vane, 1976). Tissues were superfused with Tyrode solution at 37°C (gassed with O_2) at 6 ml/min, containing indomethacin 1 μg/ml and the following antagonist mixture to improve assay specificity: dibenzamine 0.1 μg/ml, hyoscine 0.1 μg/ml, mepyramine 0.1 μg/ml, methysergide 0.2 μg/ml, propranolol 2 μg/ml and dihydroergotamine 0.5 μg/ml.

Dihydroergotamine was included when it was found that methysergide failed to abolish the vascular smooth muscle response of the rabbit aorta to the high concentration of 5-hydroxytryptamine released from rabbit platelets during collagen-induced aggregation.

Results

Collagen fibrils at a low dose (5 to 10 μg/ml) or a high dose (50 μg/ml) when added to PRP produced platelet aggregation which was accompanied by the formation of TxA_2 and prostaglandins since, when

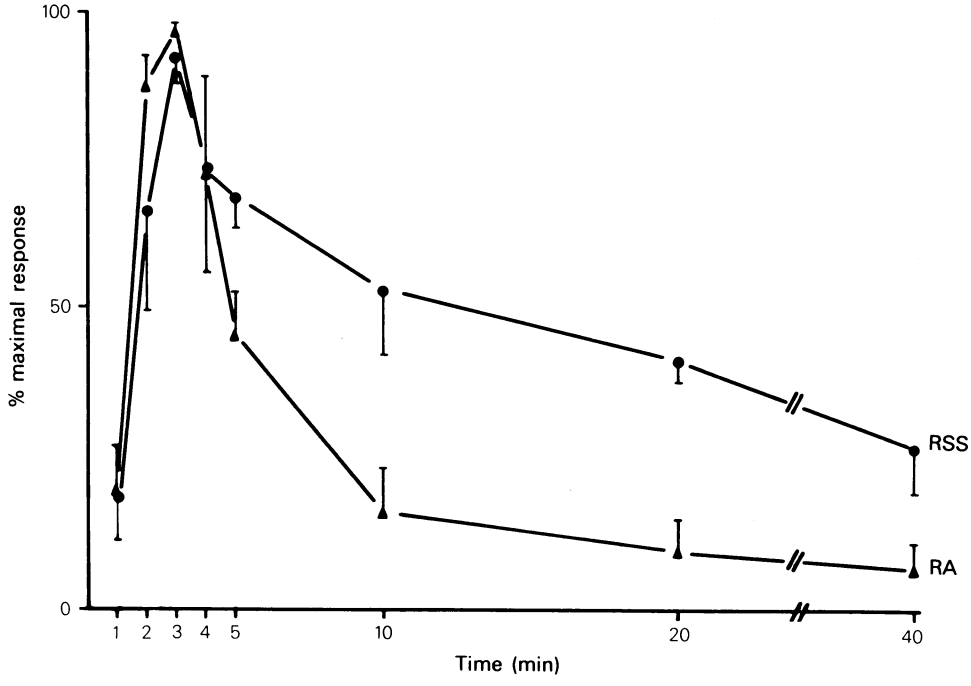

Figure 1 The time course of thromboxane A_2 (TxA_2) (rabbit aorta, RA) and prostaglandin (rat stomach strip, RSS) release from rabbit platelets aggregated with 50 μg/ml collagen fibrils. Each point represents the mean of 5 experiments in which each response was expressed as a percentage of the maximum observed in that experiment; vertical lines indicate s.e.mean.

aliquots of the platelet suspension were removed from the cuvette during aggregation and passed over the tissue cascade, both tissues gave a contraction proportional to the concentration of collagen added to PRP. Untreated PRP added directly to the tissues produced minimal or no response.

Time course

The time course of TxA_2 and prostaglandin formation from PRP activated with a high dose of collagen fibrils is illustrated in Figure 1, where peak formation of TxA_2 and prostaglandins occurred between 2 and 3 min following addition of collagen to platelets. At a low dose of collagen, the peak occurred at 3 to 4 min. The presence of RA contracting activity for a prolonged period following the initial burst of TxA_2 formation was not a measure of TxA_2 stability but probably represented a small metabolic turnover of arachidonate from the platelets remaining in contact with collagen fibrils. This was supported by experiments in which plasma was separated from platelets, following the peak formation of TxA_2 and kept at 37°C when the RA contracting activity was lost within 2 min.

Inhibition of platelet thromboxane synthetase and cyclo-oxygenase

In order to investigate the effect of inhibitors of TxA_2 and prostaglandin formation on collagen-induced platelet aggregation, the action of selected inhibitors was first examined on cyclo-oxygenase (aspirin and indomethacin) or thromboxane synthetase (imidazole derivatives). The inhibitors were added to PRP 3 min before the addition of the collagen fibrils.

The cyclo-oxygenase inhibitors gave a dose-dependent inhibition of both TxA_2 and prostaglandin formation as indicated by a concomitant reduction of responses of the RA and the RSS. The effect of indomethacin 7 μM is illustrated in Figure 2a.

The imidazole derivatives gave a dose-related reduction in the response of RA to TxA_2 which was accompanied by an enhanced response of the RSS. The effect of carboxyheptylimidazole 4 μM is illustrated in Figure 2b.

The IC_{50} for two inhibitors of cyclo-oxygenase and five inhibitors of TxA_2 synthetase, were calculated from the dose-related plots obtained from a series of such experiments and are given in Table 1.

Whilst the IC_{50} values for aspirin and indometha-

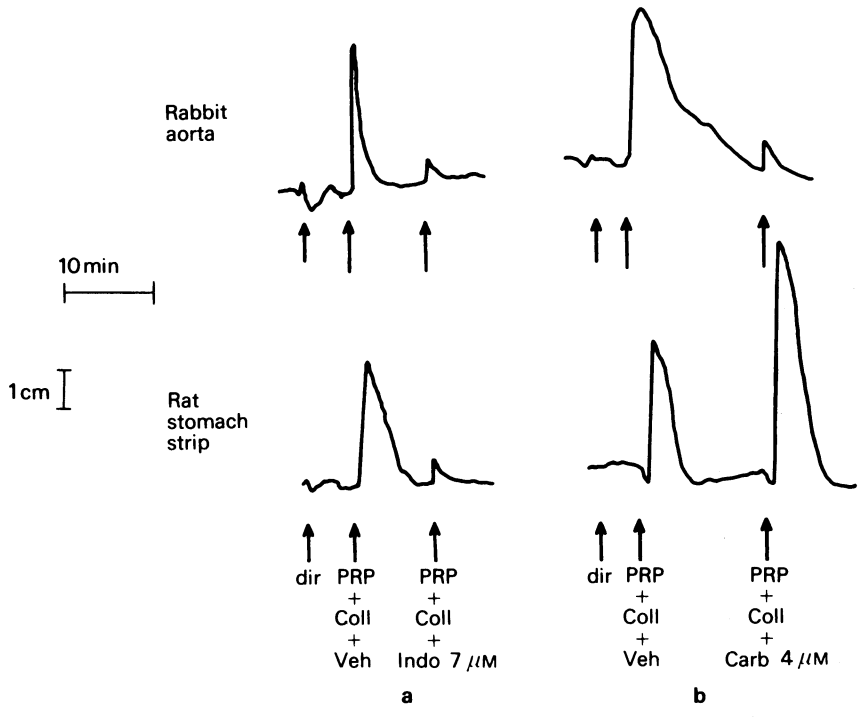

Figure 2 Responses of the rabbit aorta (RA) (upper tracing) and rat stomach strip (RSS) (lower tracing) to platelet-rich plasma (PRP) added directly to the tissue (dir) and PRP incubated for 3 min with either vehicle or indomethacin (Indo) (7 μM) or carboxyheptylimidazole (Carb) (4 μM) followed by a further 2 min incubation in the presence of collagen (Coll) (50 μg/ml). Indo inhibits responses of RA and RSS whilst Carb inhibits the response of the RA but potentiates that of the RSS.

Table 1 Inhibition of platelet thromboxane synthetase and cyclo-oxygenase

Compound	IC_{50} *Rabbit aorta* (μM)	IC_{50} *Rat stomach strip* (μM)
Aspirin	37	59
Indomethacin	3.7	3.5
Imidazole	235	*
Butylimidazole	26	*
Nonylimidazole	94	992
Clotrimazole	10	*
Carboxyheptylimidazole	0.6	*

Each IC_{50} was determined from a dose-response plot comprising the results from experiments with 6 different batches of platelets.
*No IC_{50} determination was possible for these imidazole derivatives as no inhibition of responses of the rat stomach strip was obtained over the concentration range used to obtain the IC_{50} potency estimate on the rabbit aorta.

cin were of the same order in respect of the RA and RSS, those for the TxA_2 synthetase inhibitors could only be calculated accurately in respect of RA since at most concentrations, with the exception of nonylimidazole, they potentiated responses of the RSS. These findings showed that indomethacin and carboxyheptylimidazole were specific and potent inhibitors of cyclo-oxygenase and TxA_2 synthetase respectively. Therefore, the effect of these two enzyme inhibitors was investigated on low and high dose collagen-induced platelet aggregation.

Collagen-induced platelet aggregation

High dose (50 μg/ml) collagen-induced aggregation was only partially inhibited by indomethacin and carboxyheptylimidazole as illustrated in Figure 3a, b. Both inhibitors only produced about 30% inhibition of platelet aggregation in concentrations that abolished the response of the RA to TxA_2.

In contrast, both inhibitors reduced low dose (5 μg/ml) collagen-induced aggregation in a dose-

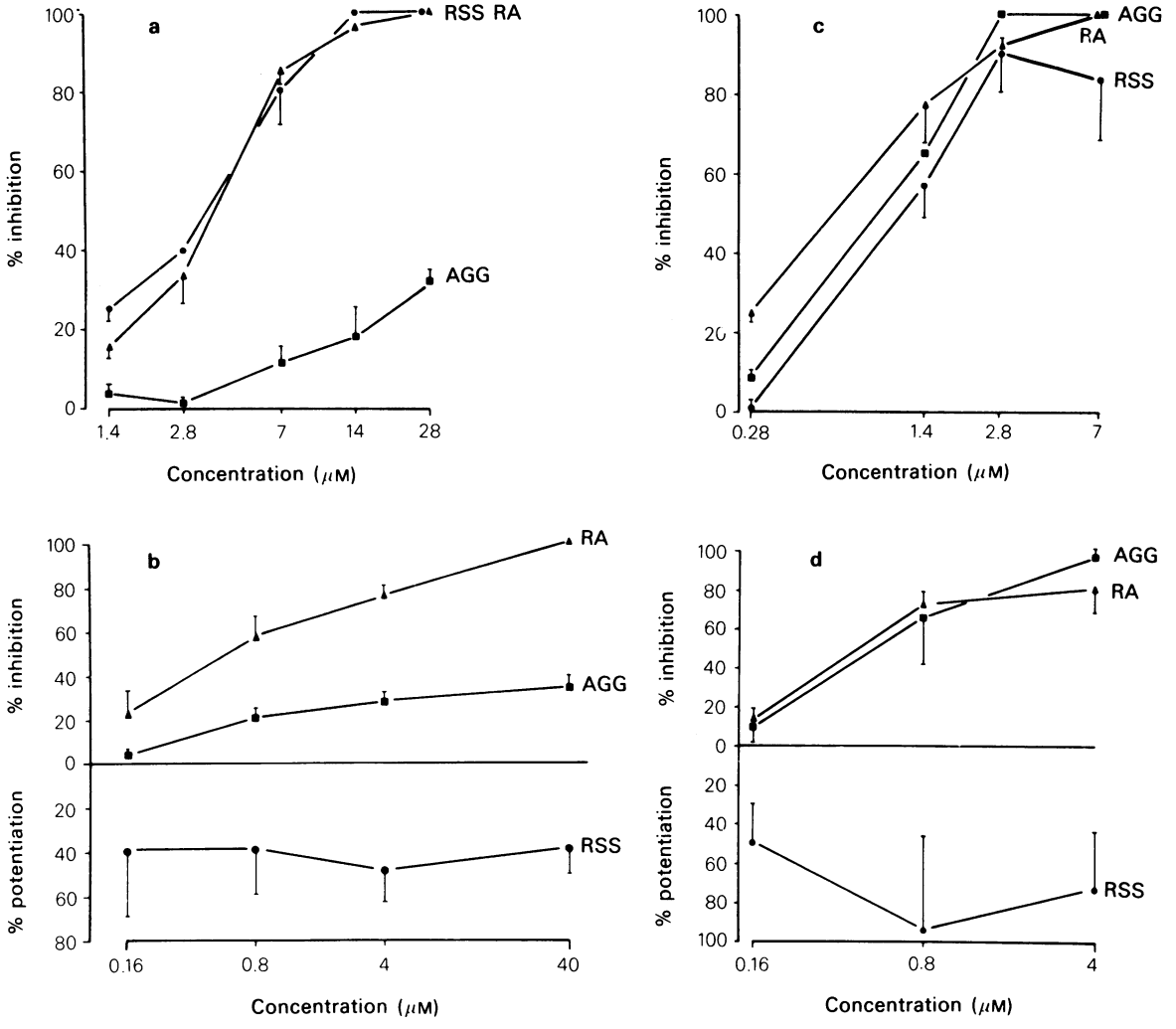

Figure 3 A comparison of the dose-related effects of indomethacin and carboxyheptylimidazole on high dose (50 μg/ml) and low dose (5 μg/ml) collagen-induced platelet aggregation and the concomitant release of thromboxane A_2 (TxA_2) and prostaglandins. (a) High dose collagen (50 μg/ml) and indomethacin (1.4–28 μM); (b) high dose collagen (50 μg/ml) and carboxyheptylimidazole (0.16–40 μM); (c) low dose collagen (5 μg/ml) and indomethacin (0.28–7 μM); (d) low dose collagen (5 μg/ml) and carboxyheptylimidazole (0.16–4 μM). Mean ($n = 6$) % inhibition or potentiation of responses of platelets incubated with collagen plus vehicle is shown; vertical lines indicate s.e.mean.

related fashion and abolished aggregation as well as the response of the RA. Furthermore, the effect on aggregation paralleled inhibition of TxA_2 formation as shown in Figure 3c, d.

Effect of creatine phosphate (CP)/creatine phosphokinase (CPK) on collagen-induced platelet aggregation

The partial inhibition of high dose collagen-induced aggregation, while cyclo-oxygenase or TxA_2 synthetase were completely inhibited, indicated the participation of an additional factor responsible for the observed aggregation. The possibility that this was adenosine diphosphate (ADP) was examined by using the substrate/enzyme complex of CP and CPK. A concentration of CP/CPK (5 mM/10 units/ml) abolished platelet aggregation to ADP (10 μM).

The complex or CP and CPK alone were added to PRP 3 min before the addition of collagen. Whilst CP (5 mM) and CPK (10 units/ml PRP) alone produced no inhibition of low or high dose collagen-induced

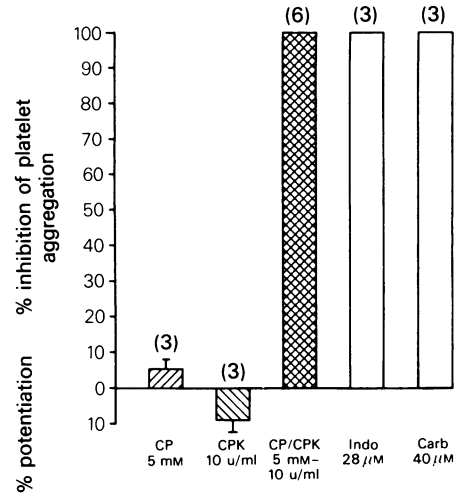

Figure 4 Effects of creatine phosphate (CP, 5 mM), creatine phosphokinase (CPK 10 u/ml), indomethacin (Indo, 28 μM) and carboxyheptylimidazole (Carb, 40 μM) on platelet aggregation induced by low dose (5 μg/ml) collagen. Results are expressed as % inhibition of aggregation where $n = 3$ or 6 as shown in parentheses and s.e.mean by vertical lines.

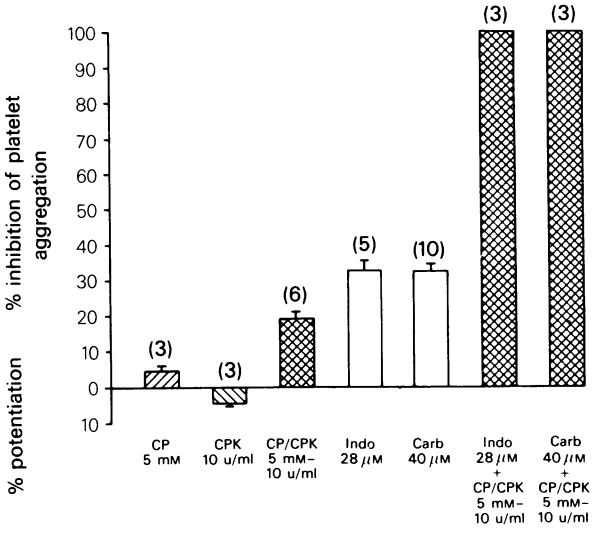

Figure 5 Effects of creatine phosphate/creatine phosphokinase (CP/CPK 5 mM; 10 u/ml) alone and in combination with indomethacin (Indo, 28 μM) or carboxyheptylimidazole (Carb, 40 μM) on platelet aggregation induced by high dose (50 μg/ml) collagen. Results are expressed as % inhibition of aggregation where n is shown in parentheses above each column and s.e.mean by vertical lines.

aggregation, the complex abolished low dose but only partially inhibited high dose collagen-induced aggregation, as illustrated in Figures 4 and 5. However, when indomethacin or carboxyheptylimidazole were used in combination with the CP/CPK complex the aggregation to high dose collagen-induced aggregation was abolished, as shown in the histogram in Figure 5.

Arachidonic acid-induced platelet aggregation

Arachidonic acid (80–800 μM) produced platelet aggregation with accompanying contraction of the RA, RSS and the RCA.

Indomethacin at a concentration (28 μM) previously found to abolish platelet cyclo-oxygenase activity, abolished AA-induced platelet aggregation and the response of RA and RSS at all concentrations. Carboxyheptylimidazole at a concentration (40 μM) previously found to abolish platelet TxA_2 synthetase activity, completely inhibited low dose (80 μM) AA-induced platelet aggregation and the response of the RA but enhanced the response of RSS. When the concentration of AA was increased, the inhibition of aggregation by carboxyheptylimidazole was surmounted so that at 320 μM AA, inhibition was $53 \pm 17\%$ ($n = 4$). The following points indicated that the remaining aggregation was due to prostaglandin endoperoxides rather than TxA_2:

(1) The aggregation was reversible in nature in contrast to the irreversible arachidonate-induced aggregation in the absence of the inhibitor.
(2) Samples of PRP from 320 μM AA/40 μM carboxyheptylimidazole produced relaxation of the RCA, a small residual contraction of the RA and an enhanced RSS response.
(3) A time course study indicated that the observed relaxation of the RCA decreased in parallel with the decrease in the residual activity on the RA (to endoperoxides), whereas the activity on the RSS was maintained.

Furthermore the CP/CPK complex in a concentration that abolished low-dose collagen-induced aggregation did not significantly inhibit platelet aggregation induced by AA (80 μM). In addition, a combination of the CP/CPK complex and carboxyheptylimidazole did not produce any more inhibition than that produced by the imidazole derivative alone.

Synthetic PGH_2 (1–10 μg/ml) added to PRP produced a rapid and reversible aggregation of platelets that was not affected by indomethacin (28 μM). The TxA_2 synthetase inhibitor, carboxyheptylimidazole at 40 μM, a concentration previously found to abolish platelet TxA_2 synthetase activity, inhibited platelet aggregation induced by PGH_2 (2.5 μg/ml) by only $11 \pm 1\%$ ($n = 3$).

Discussion

The bioassay procedure previously described (Ladd & Lewis, 1980) has been modified and used in the present experiments to determine the potency and specificity of inhibitors of platelet cyclo-oxygenase and TxA_2 synthetase. The effect of two such inhibitors, indomethacin and carboxyheptylimidazole respectively, has been examined on collagen and AA-induced platelet aggregation.

The presence of TxA_2 synthetase inhibitors in the PRP before activation by collagen or AA caused not only a reduction in the amount of TxA_2 formed, but also an increase in the amount of prostaglandins produced, as estimated by contractions of the RSS. This finding indicates that the build-up of endoperoxides resulting from blockade of the favoured metabolic route to TxA_2, leads to either chemical degradation to $PGF_{2\alpha}$ or enzymatic conversion to PGE_2, both of which are active in contracting the RSS (Bunting *et al.*, 1976). In contrast, cyclo-oxygenase inhibitors reduced the RSS contraction and this differential response of the stomach strip enabled cyclo-oxygenase and thromboxane synthetase inhibitors to be distinguished. Furthermore, inhibition of the RSS by the imidazole derivatives reflected a low specificity for the TxA_2 synthetase, as illustrated for nonylimidazole, a result in agreement with the findings of Prancan, Lefort, Chignard, Gerozissis, Dray & Vargaftig (1979).

The results of this investigation, lead to the conclusion that the mechanism of collagen-induced aggregation of rabbit platelets *in vitro* is mediated by both TxA_2 formation and ADP release. It is probable that both mediators are involved since, when a low dose of collagen is used, either inhibition of TxA_2 formation or inactivation of released ADP by the substrate/enzyme complex CP/CPK can completely inhibit platelet aggregation, indicating that subthreshold levels of released ADP and TxA_2 can act synergistically to induce platelet aggregation. However, with increasing concentration of collagen, sufficient ADP and TxA_2 are released to induce aggregation independently of each other since, abolition of TxA_2 formation or the CP/CPK catalysed breakdown of ADP produced only partial inhibition of aggregation when used alone, whilst in combination aggregation was abolished.

Our conclusion is consistent with the finding of Silver, Smith, Ingerman & Kocsis (1973) who observed that arachidonic acid, in concentrations too low to cause aggregation enhanced platelet aggregation induced by collagen or ADP. Our results are also in agreement with those of Kinlough-Rathbone *et al.* (1977a, b) that synergism occurs between products of the arachidonate pathway and other release-inducing agents such as thrombin.

The response of rabbit platelets to ADP is not complicated by second phase aggregation as occurs with human platelets (Thomas, Niewiarowski & Ream, 1970). This species difference is important when considering the synergistic effect to which released ADP contributes in the results we have described. The contribution of ADP in collagen-induced aggregation has been evaluated in our study using the CP/CPK complex to breakdown ADP released from platelets, as has been used previously (Haslam, 1964; 1967; Massini & Lüscher, 1972). However, caution in interpretation of results using such nucleotide-degrading enzymes has been suggested (Nunn, 1979) although this is not supported either by the fact that collagen-induced aggregation of thrombin degranulated platelets (devoid of releasable ADP) is unaffected by CP/CPK (Reimers, Kinlough-Rathbone, Cazenave, Senyi, Hirsh, Packham & Mustard, 1976) or our findings that CP or CPK alone have no inhibitory effect on collagen or ADP-induced platelet aggregation. More recently, Huang & Detwiler (1980) have proposed an alternative hypothesis for the mechanism of action of CP/CPK and the involvement of secreted ADP in platelet aggregation. They propose that inhibition of platelet aggregation with CP/CPK cannot be taken as evidence for the involvement of secreted ADP and that inhibition is a consequence of a decrease in potentiation by extracellular ADP present in plasma before addition of a stimulus. CP/CPK has, however, been shown to inhibit collagen-induced aggregation of washed intact platelets where any extracellular ADP has been removed (Kinlough-Rathbone, Cazenave, Packham & Mustard, 1980 .

In contrast to collagen, AA-induced platelet aggregation did not depend upon ADP release since CP/CPK did not inhibit aggregation, a finding supported by experiments with thrombin degranulated rabbit platelets and arachidonate (Kinlough-Rathbone, Reimers, Mustard & Packham, 1976) but in contrast to the report of Charo *et al.* (1977) that AA-induced aggregation was always accompanied by secretion. A possible explanation for the apparent discrepancy in these results is that the quantity of ADP released during arachidonate-induced aggregation is insufficient to modulate significantly the aggregatory response following formation of the prostaglandin endoperoxides and TxA_2.

In examining the arachidonate-dependent pathway of collagen-induced platelet aggregation, we wished to determine the relative importance of the prostaglandin endoperoxides compared to TxA_2 in mediating platelet aggregation. Both indomethacin and carboxyheptylimidazole abolished low dose collagen-induced aggregation, indicating that endogenously formed prostaglandin endoperoxides do not play any direct pro-aggregatory role in platelet

aggregation induced by collagen.

This prompted us to replace collagen with AA as a platelet aggregating agent, to assist in resolving the relative contributions of TxA_2 and/or endoperoxides to platelet aggregation, a question which has produced conflicting answers (Raz *et al.*, 1977; Fitzpatrick & Gorman, 1978). Our experiments showed that whilst indomethacin completely inhibited AA-induced aggregation, carboxyheptylimidazole only abolished aggregation induced by low concentrations of arachidonate. However, with increasing AA concentration this inhibition of aggregation was surmounted and furthermore was reversible in nature, in contrast to the normally irreversible aggregation produced in the absence of the TxA_2 synthetase inhibitor. These results indicate that at low arachidonate concentrations, TxA_2 formation appears to be responsible for platelet aggregation whilst in the absence of TxA_2 formation, a high concentration of arachidonate is converted by the platelet cyclooxygenase to produce endoperoxides in sufficient quantity to directly aggregate the platelets.

This proposal is supported by bioassay results where carboxyheptylimidazole-treated PRP, aggregated with arachidonic acid, produced relaxation of the rabbit coeliac artery, in contrast to the contraction to TxA_2 observed in the absence of the inhibitor. Although it is conceivable that some of the observed relaxation of the coeliac artery could be attributed to the increase in prostaglandins as a consequence of Tx synthetase inhibition, the time-course of such relaxation indicated that endoperoxides were responsible for this effect.

The conflicting evidence in support of the prostaglandin endoperoxides and/or TxA_2 as prime mediators in propagating platelet aggregation probably reflects differences between the metabolism of exogenous or endogenous arachidonate substrate.

Exogenous PGH_2 (the endoperoxide available to us) added to PRP produced only reversible platelet aggregation which was only partially inhibited by the presence of carboxyheptylimidazole and unaffected by indomethacin. It is possible that PGH_2 is not readily converted to TxA_2 in PRP as occurs in washed platelet suspensions (Needleman *et al.*, 1976). Another possible explanation for the TxA_2 synthetase inhibitor having little effect upon PGH_2-induced aggregation is that PGG_2 has been shown to be three times more potent than PGH_2 as a platelet aggregant (Hamberg, Svensson, Wakabayashi & Samuelsson, 1974) so that the latter may not be the most favoured substrate for TxA_2 synthetase.

The present findings suggest that collagen-induced aggregation of rabbit platelets is normally mediated by a combination of TxA_2 and ADP. AA-induced aggregation is normally mediated by TxA_2 and only when TxA_2 formation is prevented do the endoperoxides play a significant role.

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