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The roles of prostaglandin endoperoxides, thromboxane A_2 and adenosine diphosphate in collagen-induced aggregation in man and the rat

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1 The effects of aspirin, carboxyheptylimidazole (CHI) and creatine phosphate/creatine phosphokinase (CP/CPK) on platelet aggregation and thromboxane B_2 (TxB_2) formation induced by collagen have been examined *in vitro*. Platelets from two species, man and the rat, have been used.

2 In man, aspirin and CHI abolished TxB_2 production but only partially inhibited aggregation. CP/CPK partially inhibited aggregation and TxB_2 formation.

3 In the rat, aspirin and CHI abolished TxB_2 formation but had no effect on aggregation. CP/CPK completely inhibited aggregation and partially inhibited TxB_2 generation.

4 In man, collagen-induced aggregation is largely dependent on ADP and to a lesser extent on arachidonate metabolites whereas, in the rat, ADP alone mediates aggregation induced by this agonist.

5 The results with CP/CPK suggest that TxB_2 formation is dependent either on the prior release of platelet ADP or on aggregation itself rather than being responsible for the aggregation response.

Introduction

Pro-aggregatory adenosine diphosphate (ADP) (Hovig, 1963), prostaglandin endoperoxides (PGG_2, PGH_2) (Smith *et al.*, 1974) and thromboxane A_2 (TxA_2) (Malmsten *et al.*, 1975) are known to be released from platelets following collagen stimulation and yet their relative contribution to an event which may be of prime importance in the formation of the haemostatic plug remains unclear. In 1963, Hovig proposed that the ADP released may be responsible for aggregation induced by collagen. However, collagen may induce aggregation in degranulated platelets devoid of releasable ADP (Kinburgh-Rathbone *et al.*, 1980) and also in platelets rendered unresponsive to ADP (Kinlough Rathbone *et al.*, 1977). Similarly, blocking the conversion of arachidonate to PGG_2, PGH_2 and TxA_2, by aspirin, does not prevent aggregation induced by high concentrations of collagen (Kinburgh-Rathbone *et al.*, 1980).

We have investigated further the contribution of ADP, PGG_2/PGH_2 and TxA_2 in platelet aggregation induced by collagen. We have used platelets from man and the rat, since it has become apparent that the role of each of these mediators may vary in different species. Whereas, in most species, platelets aggregate in response to endoperoxides and TxA_2, in the dog for example, although platelets form both endoperoxides and TxA_2 in the presence of arachidonate, they do not aggregate (Chignard *et al.*, 1976).

We have previously used a superfusion cascade system, comprising the rabbit aorta and the rat stomach strip, to monitor qualitatively TxA_2 and prostaglandin release from rabbit platelets following collagen stimulation (Lewis & Watts, 1982). In the present investigation, we have monitored aggregation and also quantitated TxA_2 production in the same platelet sample, by means of a radioimmunoassay specific for TxB_2, the stable metabolite of TxA_2. We have used the substrate/enzyme complex creatine phosphate/creatine phosphokinase (CP/CPK), which converts ADP to ATP (Kinlough-Rathbone *et al.*, 1980), to assess the contribution of ADP. Furthermore, aspirin has been used to inhibit cyclo-oxygenase activity and thus determine the role of arachidonate metabolites PGG_2/PGH_2 and TxA_2, while carboxyheptylimidazole (CHI), a potent inhibitor of thromboxane synthetase (Butler *et al.*, 1982), has been used to identify the contribution of TxA_2 alone in collagen-induced platelet aggregation.

Methods

Preparation of platelet-rich plasma (PRP)

Male human blood was collected into tri-sodium citrate (3.8% final blood concentration) and PRP prepared as previously described (Westwick & Webb, 1978). Blood from male Wistar rats (350–450 g body weight) was collected into heparin (5 iu ml^{-1} final blood concentration) and PRP obtained as described earlier (Emms & Lewis, 1985). PRP from 2 or 3 rats was pooled for each experiment. The platelet count of human and rat PRP was adjusted to 3×10^8 ml^{-1} and 8×10^8 ml^{-1} respectively, with autologous platelet-poor plasma (PPP) and stored at room temperature in capped plastic pots prior to use.

Platelet aggregation

Platelet aggregation was carried out in a Born MkIII aggregometer. PRP was equilibrated at 37°C for 2 min in the aggregometer cuvette before the addition of drug or vehicle. Collagen (Hormon Chemie) was added 2 min later and aggregation monitored for a further 8 min. The sample was then mixed with indomethacin (1.4 mM final concentration) and centrifuged at 10,000 *g* for 1 min. The supernatant PPP was then removed and rapidly frozen in solid CO_2 before extraction and radioimmunoassay.

All drug concentrations given are their final concentrations in PRP. Carboxyheptylimidazole was a gift from Dr R. Wallis of Ciba-Geigy, Horsham. Where the substrate/enzyme complex CP/CPK (Sigma Chemical Co) was used, the concentration of CPK was kept constant (10 u ml^{-1}), while that of CP was varied.

Extraction and radioimmunoassay for thromboxane B_2

The pH of PPP (0.4 ml) was adjusted to 3–3.5 by the addition of 1 M citric acid. TxB_2 was extracted twice with 2 volumes of ethyl acetate. The first organic phase was collected by centrifugation at 250 *g* for 10 min and combined with the second organic phase, collected by centrifugation at 100 *g* for 10 min. The combined phases were evaporated to dryness overnight in a fume cupboard. The extracts were stored at −20°C and reconstituted in Tricine buffered saline (TBS) for radioimmunoassay. The recovery of added [^{3}H]-TxB_2 was 60 ± 2% from human PPP and 67 ± 1% from rat PPP after extraction.

The radioimmunoassay method used was that described by Hennam *et al.* (1974) with the modifications of Jose *et al.* (1981). Antiserum to TxB_2 was prepared by L. Levine, Brandeis University, Mass, U.S.A. and was a gift from J.F. Parry, Pfizer UK Ltd, Sandwich. The antiserum was diluted (1:20,000) to give 40–50% binding with [^{3}H]-TxB_2 in the absence of non-radioactive TxB_2. The cross-reactivity of this antiserum with other arachidonate metabolites was <0.01 except with PGD_2 (0.5%) as previously reported by Siess *et al.* (1981). The sensitivity of the assay was 20 pg. [^{3}H]-TxB_2 was purchased from New England Nuclear (specific activity 100–150 G $mmol^{-1}$) and diluted to give 4×10^4 dpm per assay tube. Non-radioactive TxB_2 was obtained from Upjohn Diagnostics, Kalamazoo, Michigan, U.S.A.

Statistics

All values are expressed as mean ± standard error of mean (s.e.mean).

Results

Human platelet aggregation and thromboxane formation induced by collagen

Collagen induced a concentration-dependent increase in the extent of aggregation and TxB_2 generated by human platelets (Figure 1). However, platelet aggregation was maximal at lower concentrations of collagen (1 μg ml^{-1} collagen final concentration in PRP) than

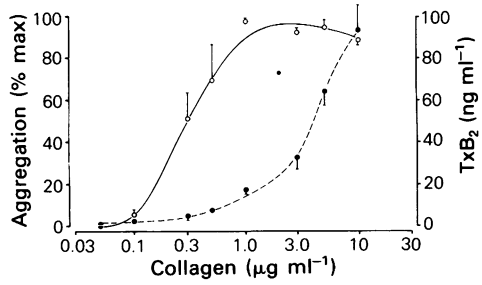

Figure 1 Human platelet aggregation and thromboxane B_2 (TxB_2) formation induced by collagen *in vitro*. Platelet aggregation (continuous line) and TxB_2 formation (broken line) were monitored in the same aliquot of platelet-rich plasma (PRP). The maximum height of the aggregation response reached within 8 min of adding collagen was determined and expressed as a percentage of the maximum height induced by 10 μg ml^{-1} collagen. TxB_2 was measured by radioimmunoassay following ethyl acetate extraction of the 8-min sample. Concentrations of TxB_2 shown have been corrected for extraction losses. All concentrations shown are the final concentrations in PRP. Each point represents the mean of 3 experiments and vertical lines show s.e.mean where greater than the symbols. The height of the aggregation response and the amount of TxB_2 generated by human platelets was dependent on the collagen concentration. However, maximal aggregation was achieved when TxB_2 formation was minimal.

those required to induce maximal TxB_2 formation (greater than 10 $\mu g\,ml^{-1}$ collagen). The level of TxB_2 in unstimulated PRP was $0.8 \pm 0.5\,ng\,ml^{-1}$ and increased to $4.8 \pm 2.2\,ng\,ml^{-1}$ TxB_2 at 0.3 $\mu g\,ml^{-1}$ collagen when aggregation was maximal. At 10 $\mu g\,ml^{-1}$ collagen, $93.5 \pm 11.9\,ng\,ml^{-1}$ TxB_2 was formed, demonstrating that whereas aggregation was maximal at 1 $\mu g\,ml^{-1}$ the capacity to produce TxB_2 was not. A concentration of collagen, 3 $\mu g\,ml^{-1}$, which produced maximal platelet aggregation and the formation of $32.7 \pm 5.8\,\mu g\,ml^{-1}$ TxB_2, was chosen to study the effects of inhibitors.

The effects of aspirin, carboxyheptylimidazole and creatinine phosphate/creatine phosphokinase on human platelet aggregation and thromboxane formation induced by collagen

Aspirin produced a concentration-dependent inhibition of TxB_2 formation as shown in Figure 2, the concentration of aspirin required to produce 50% inhibition of TxB_2 formation (IC_{50}) being 0.72 mM (final concentration in PRP). However, at concentrations of aspirin (1 mM) which abolished TxB_2 formation, platelet aggregation was inhibited by only 40%. The maximum inhibition of human platelet aggregation, at the concentration of collagen used, was $46 \pm 5\%$ with 10 mM aspirin.

Carboxyheptylimidazole (CHI) similarly produced a concentration-dependent inhibition of TxB_2 formation induced by collagen (Figure 3). The IC_{50} for inhibition of TxB_2 formation by CHI was 0.76 mM. However, at concentrations of CHI (10 mM) which inhibited TxB_2 formation by over 90%, platelet aggregation was reduced only by approximately 20%.

Creatine phosphate/creatine phosphokinase inhibited platelet aggregation and thromboxane generation induced by collagen in a manner dependent on the concentration of CP (substrate) used, the enzyme (CPK) concentration being kept constant at 10 u ml^{-1} (Figure 4). The maximum inhibition of platelet aggregation (60%) was seen at a concentration of CP (10 mM) which also maximally inhibited TxB_2 generation by almost 50%.

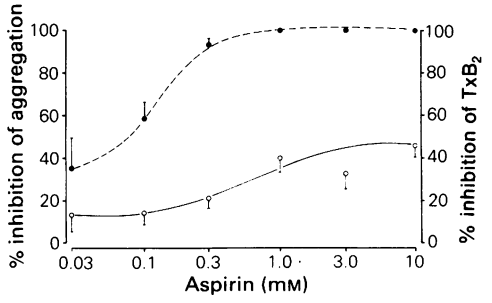

Figure 2 The effect of aspirin on human platelet aggregation and thromboxane B_2 (TxB_2) generation induced by collagen 3 $\mu g\,ml^{-1}$. Platelet aggregation and TxB_2 formation were monitored as described for Figure 1. PRP was pre-incubated with aspirin (final concentration shown) for 2 min prior to the addition of collagen. Inhibition of aggregation (continuous line) and TxB_2 formation (broken line) produced by aspirin are expressed as a percentage of responses in the presence of vehicle and collagen only. Each point represents the mean of 4 experiments and vertical lines show s.e.mean where greater than the symbols. Aspirin produced a concentration-dependent inhibition of TxB_2 formation but only inhibited aggregation by up to 46%.

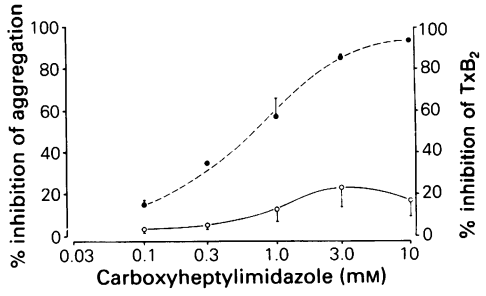

Figure 3 The effect of carboxyheptylimidazole (CHI) on human platelet aggregation and thromboxane B_2 (TxB_2) generation induced by collagen 3 $\mu g\,ml^{-1}$. Pre-incubation of platelet-rich plasma (PRP) with CHI and assessment of the inhibition of aggregation (continuous line) and TxB_2 formation (broken line) were carried out as described for Figure 2. Each point represents the mean of 3 experiments and vertical lines show s.e.mean where greater than the symbols. CHI produced a concentration-dependent inhibition of TxB_2 formation but only reduced aggregation-stimulated by collagen by approximately 20%.

Rat platelet aggregation and thromboxane formation induced by collagen

Both the extent of platelet aggregation and the amount of TxB_2 formed by stimulated rat PRP was dependent on the concentration of collagen used (Figure 5). As seen for human platelets, the amount of collagen required to produce maximal aggregation was much lower than that necessary to produce maximal TxB_2 formation. The basal amount of TxB_2 in non-activated rat PRP was $0.12 \pm 0.03\,ng\,ml^{-1}$. Collagen 10 $\mu g\,ml^{-1}$ induced the formation of $14.4 \pm 0.2\,ng\,ml^{-1}$ TxB_2 and maximal platelet aggregation, whereas even higher collagen concentrations (30 $\mu g\,ml^{-1}$) were able to induce the formation of even more TxB_2 ($40.3 \pm 4\,ng\,ml^{-1}$). Rat platelets were, however, less sensitive to collagen than human, the threshold concentration

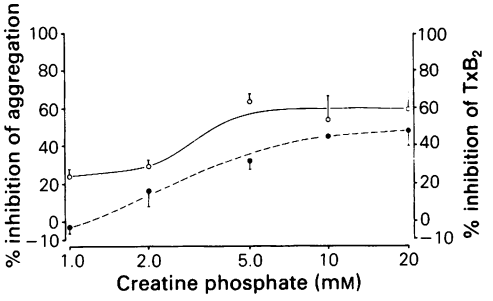

Figure 4 The effect of creatine phosphate (CP)/creatine phosphokinase (CPK) on human platelet aggregation and thromboxane B_2 (TxB_2) generation induced by collagen 3 $\mu g\,ml^{-1}$. Human platelet-rich plasma (PRP) was pre-incubated with CP/CPK for 2 min prior to the addition of collagen. The concentration of CPK used was 10 u ml^{-1} while that of CP was varied, as shown on the abscissa scale (final concentration in PRP shown). Assessment of the inhibition of aggregation (continuous line) and TxB_2 formation (broken line) has been described in Figure 2. Each point represents the mean of 3 experiments and vertical lines show s.e.mean where greater than the symbols. The combination CP/CPK inhibited collagen-induced human platelet aggregation by up to 60% and TxB_2 generation by almost 50%.

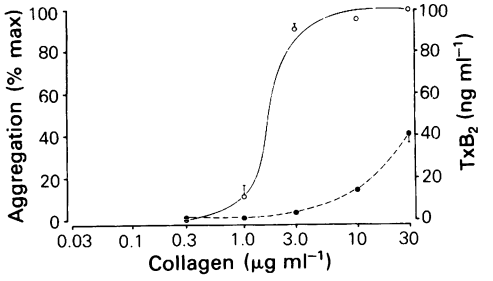

Figure 5 Rat platelet aggregation and thromboxane B_2 (TxB_2) formation induced by collagen *in vitro*. The aggregation of rat platelets was monitored for 8 min following the addition of collagen. The platelet-rich plasma (PRP) was then removed, extracted with ethyl acetate and TxB_2 determined by radioimmunoassay. The height of the aggregation response (continuous line) is expressed as a percentage of the maximum height of aggregation induced by 30 $\mu g\,ml^{-1}$ collagen. The amounts of TxB_2 formed (broken line) have been corrected for extraction losses and are the final concentrations in PRP. Each point represents the mean of 3 experiments and vertical lines show s.e.mean where greater than the symbols. Both the extent of aggregation and the amount of TxB_2 formed by rat platelets were dependent on the collagen concentration. Rat platelet aggregation was, however, maximal at lower concentrations of collagen than those required to induce maximal TxB_2 formation.

required to produce aggregation in rat PRP was 0.3–1 $\mu g\,ml^{-1}$ in contrast to 0.05–1 $\mu g\,ml^{-1}$ in human PRP (although platelet counts were adjusted to $8 \times 10^8\,ml^{-1}$ in rat PRP and $3 \times 10^8\,ml^{-1}$ in human PRP). To determine the effects of drugs on rat platelet activation, a concentration of collagen that produced maximal aggregation was used (5 $\mu g\,ml^{-1}$).

The effects of aspirin, carboxyheptylimidazole and creatine phosphate/creatine phosphokinase on rat platelet aggregation and thromboxane formation induced by collagen

Aspirin produced a dose-dependent inhibition of TxB_2 formation with little or no effect on rat platelet aggregation induced by collagen (Figure 6). The IC_{50} for inhibition of TxB_2 by aspirin was 0.05 mM. At concentrations of aspirin which inhibited TxB_2 by 98% (0.3 and 1.0 mM aspirin) aggregation was inhibited by only 5%.

Carboxyheptylimidazole also inhibited TxB_2 formation by collagen-stimulated rat platelets in a concentration-dependent manner (Figure 7). The IC_{50} for inhibition of TxB_2 by CHI was 2.0 mM, but again even higher concentrations (up to 200 mM CHI) had no effect on rat platelet aggregation.

Creatine phosphate/creatine phosphokinase, on the other hand, inhibited both rat platelet aggregation and TxB_2 formation induced by collagen (Figure 8). In the presence of CPK 10 u ml^{-1}, increasing concentrations of CP produced a concentration-dependent inhibition of aggregation of up to 97% and inhibition of TxB_2 of

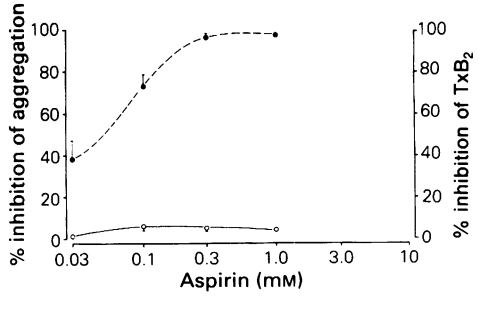

Figure 6 The effect of aspirin on rat platelet aggregation and thromboxane B_2 (TxB_2) generation induced by collagen 5 $\mu g\,ml^{-1}$. Pre-incubation of rat platelet-rich plasma (PRP) with aspirin and assessment of the inhibition of collagen-induced aggregation (continuous line) and TxB_2 generation (broken line) were carried out as described in Figure 2. Each point represents the mean of 3 experiments and vertical lines show s.e.mean where greater than the symbols. Aspirin produced a concentration-dependent inhibition of TxB_2 formation with little or no effect on collagen-induced rat platelet aggregation.

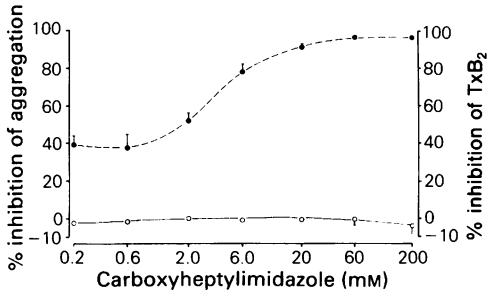

Figure 7 The effect of carboxyheptylimidazole (CHI) on rat platelet aggregation and thromboxane B_2 (TxB_2) generation induced by collagen 5 $\mu g\,ml^{-1}$. Pre-incubation of rat platelet-rich plasma (PRP) with CHI and assessment of the inhibition of collagen-induced aggregation (continuous line) and TxB_2 generation (broken line) were carried out as described in Figure 2. Each point represents the mean of 3 experiments and vertical lines show s.e.mean where greater than the symbols. CHI inhibited collagen-induced TxB_2 formation by rat platelets but had no effect on platelet aggregation.

up to 68%. The concentration of CP/CPK required to produce 50% inhibition of aggregation was 2.8 mM CP and 10 u ml^{-1} CPK. At these concentrations, CP/CPK inhibited TxB_2 formation by approximately 35%.

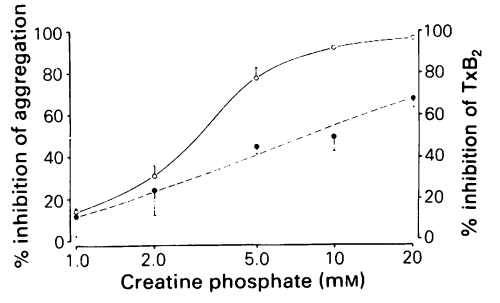

Figure 8 The effect of creatine phosphate (CP)/creatine phosphokinase (CPK) on rat platelet aggregation and thromboxane B_2 (TxB_2) generation induced by collagen 5 $\mu g\,ml^{-1}$. Rat platelets were pre-incubated with CPK 10 u ml^{-1} and CP at varying concentrations (shown on the abscissa scale) and inhibition of collagen-induced aggregation (continuous line) and TxB_2 formation (broken line) quantitated as described in Figure 2. Each point represents the mean of 4 experiments and vertical lines show s.e.mean where greater than the symbols. The combination CP/CPK inhibited collagen-induced rat platelet aggregation by up to 97% and TxB_2 generation by up to 68% in a manner dependent on the concentration of CP used.

Discussion

Human platelet aggregation induced by collagen was accompanied by the formation of TxB_2, the levels measured here being similar to those previously reported (Siess *et al.*, 1981; Best *et al.*, 1980; Krishnamurthi *et al.*, 1984). While the amount of TxB_2 formed was dependent on the collagen concentration, the capacity of human platelets to produce TxB_2 was much greater than that required to induce maximal aggregation, as noted by Siess *et al.* (1981).

The aggregation response of human platelets to collagen was only partially dependent on arachidonate metabolites. Pre-incubation of human PRP with aspirin, a cyclo-oxygenase inhibitor, abolished TxB_2 formation but only inhibited aggregation induced by collagen by approximately 40%. Thus collagen-induced aggregation occurred even in the presence of aspirin. This finding agrees with that of Best *et al.* (1980) who found that only aggregation induced by low concentrations of collagen was inhibited by aspirin and that, at high concentrations (4 $\mu g\,ml^{-1}$ collagen), aggregation still occurred even in the absence of TxB_2 formation. Furthermore, in the present study, pre-incubation of human PRP with the thromboxane synthetase inhibitor CHI which completely suppressed TxB_2 generation, inhibited aggregation by only 20%. Best *et al.* (1980) similarly found that the thromboxane synthetase inhibitor, N-butyl imidazole, abolished TxB_2 formation but only inhibited aggregation by 5%, at high collagen concentrations. We therefore conclude from our results that while TxB_2 is formed, it is not essential for the aggregation of human platelets by collagen; at most it may only be responsible for 20% of the aggregation response. The difference between the degree of inhibition of aggregation produced by aspirin (40%) and that of CHI (20%) may reflect the equally small contribution (20%) of the endoperoxides to the aggregation response induced by collagen.

Pre-incubation of human PRP with CP/CPK, on the other hand, inhibited collagen-induced platelet aggregation by 60%. This substrate/enzyme complex converts ADP to ATP, thus removing released ADP from the vicinity of the platelet (Kinlough-Rathbone *et al.*, 1980). Its effect on collagen-induced aggregation of human platelets would suggest a greater role for ADP in this response than those played by TxA_2 or PGG_2/PGH_2 either individually or combined. Furthermore, CP/CPK also inhibited TxB_2 production by 50%. Since inhibition of TxB_2 (using CHI) by 50% inhibited aggregation by less than 10% this action alone cannot account for the 60% inhibition seen with CP/CPK. It does, however, suggest that TxB_2 formation is in some way dependent on the released ADP or, alternatively, that it occurs as a result of aggregation rather than being responsible for aggregation.

Two pathways, ADP release and arachidonate metabolism, would thus appear to mediate largely the aggregation response of human platelets to collagen as concluded by Kinlough-Rathbone *et al.* (1977). The present study shows that the relative contribution of these mediators in human platelet aggregation induced by collagen could be quantitated as ADP 60%, endoperoxides 20% and TxA_2 20%.

In rat platelets, as in human, thromboxane formation occurred following collagen stimulation. Again the amounts formed were dependent on the collagen concentration but the capacity to produce TxB_2 far exceeded that required to induce maximal aggregation. Rat platelets were, however, less sensitive to collagen than human, requiring higher concentrations of collagen to induce threshold aggregation and also producing less TxB_2 at any given collagen concentration.

Although metabolites of arachidonic acid are produced by rat platelets following collagen stimulation, they do not appear to play any role in the aggregation response in this species. Aspirin and CHI both abolished TxB_2 formation but had essentially no effect on aggregation. This finding agrees with previous work in the rat using different techniques. Following the oral administration of CHI to rats, Butler *et al.* (1982) also found that platelet aggregation induced by collagen *in vitro* was unaffected. TxB_2 formation was not, however, completely inhibited in these experiments. Vincent & Zijlstra (1977) demonstrated that rat platelets incubated with phospholipase A_2 formed TxA_2 but did not aggregate in response to it.

In the rat, ADP would appear to be wholly responsible for aggregation induced by collagen. CP/CPK also, however, inhibited TxB_2 production by 70%, although this action could not be responsible for the effect seen on aggregation since CHI also abolished TxB_2 synthesis without affecting aggregation. Again, this result suggests that TxB_2 formation is in some way dependent on aggregation or perhaps on the prior release of ADP from aggregating platelets.

The results presented here, in agreement with earlier studies in rabbits, demonstrate a distinct species difference in the mechanism by which collagen induces platelet aggregation. Earlier, Lewis & Watts (1982) showed that aggregation of rabbit platelets induced by a low concentration of collagen was dependent on a 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. It was concluded that endoperoxides did not play a role in collagen-induced platelet aggregation in rabbits. In the present study, collagen-induced platelet aggregation in man was found to be dependent upon ADP and to a lesser extent upon endoperoxides and TxA_2 whereas, in the rat, it is wholly dependent on released ADP and is independent of arachidonate metabolites. The findings further suggest that TxB_2 formation may occur as a result of aggregation rather than being responsible for aggregation and may therefore be more important in other aspects of haemostasis, for example in causing vasoconstriction.

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