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Potentiation of ADP-induced aggregation in human platelet-rich plasma by 5-hydroxytryptamine and adrenaline

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1 We have used dose-response curves to quantitate the potentiation of adenosine 5'-diphosphate (ADP)-induced aggregation and thromboxane (TXA_2) generation by 5-hydroxytryptamine (5-HT) and adrenaline in human citrated platelet-rich plasma. We have also quantitated the inhibition of these responses by aspirin, ketanserin and yohimbine, singly and in pairs.

2 Ketanserin (5 μM) inhibited TXA_2 production and the second wave of platelet aggregation induced by a range of concentrations of ADP alone. This indicates that endogenous 5-HT, released from the platelet dense granules, contributes significantly to responses induced by ADP.

3 When 5-HT (10 μM) was added before ADP, a lower concentration of ADP was required to cause 50% aggregation and TXA_2 generation. The ratio of ADP concentrations (CR) to cause 50% aggregation in the presence and absence of 5-HT was 2.1 when only added 5-HT was considered, and 5.0 when endogenous 5-HT was also taken into account.

4 Potentiation of ADP-induced aggregation by 5-HT also occurred in the presence of aspirin, resulting in a CR of 2.3. As expected, ketanserin inhibited potentiation by 5-HT in the presence and absence of aspirin. Although aspirin caused substantial inhibition of aggregation induced by ADP and 5-HT (CR 3.4), further inhibition occurred when ketanserin was also present (CR 6.5).

5 A subthreshold concentration of adrenaline (0.25 μM) caused substantial potentiation of ADP-induced aggregation in the absence (CR 4.0) and presence (CR 2.0) of aspirin. As expected, yohimbine (9 μM) inhibited this potentiation. Maximum TXA_2 generation induced by ADP increased from 32.5 to 59.4 pg per 10^6 platelets when adrenaline was present. Aggregation induced by ADP and adrenaline was markedly inhibited by aspirin (CR 5.1) but was further inhibited when yohimbine (9 μM) was also present (CR 10.0).

6 Results from this *in vitro* study show ketanserin and yohimbine have the potential to be used in combination with aspirin as antithrombotic agents *in vivo*.

Keywords: Platelet aggregation; thromboxane; 5-hydroxytryptamine; adrenaline; synergism; aspirin; ketanserin; yohimbine; *in vitro*

Introduction

Platelet activation, at a site of vascular injury, by agonists such a collagen, is a receptor-mediated process. A number of biochemical events, including the hydrolysis of phosphatidylinositolbisphosphate, a rise in cytosolic calcium, synthesis of thromboxane (TXA_2) and secretion of adenosine 5'-diphosphate (ADP), 5-hydroxytryptamine (5-HT) and catecholamines from platelet dense granules, are involved, culminating in irreversible platelet aggregation. TXA_2 and 5-HT provide an important mechanism for the enhancement of platelet aggregation, and also contribute to platelet derived vasoconstriction (Mehta, 1990). Single platelet agonists have been classified according to potency with respect to which of the biochemical events are elicited. Adrenaline, 5-HT and ADP when used singly, induce platelet activation with a weak potency and irreversible aggregation will ensue only if TXA_2 synthesis occurs. Relatively few platelets actually adhere to exposed collagen at the site of vessel injury, and therefore the major fraction of platelets are probably activated by ADP, 5-HT, and TXA_2 released from platelets that interact directly wtih the collagen (Holmsen, 1982). It has been demonstrated *in vitro* that 5-HT and catecholamines released from the platelet, potentiate not only the aggregation response initiated by thrombin (Thomas, 1967), but can also potentiate the effects of released ADP (Mills *et al.*, 1968). It is thought that the function of the released agonists *in vivo*, is to potentiate platelet responses induced by other agonists in the milieu of a forming thrombus (De Clerck & De Chaffoy de Courcelles, 1990). It is conceivable that receptor antagonists such as ketanserin or yohimbine, which compete with 5-HT and catecholamines for respective receptor sites, may have a role in the prophylaxis of thrombosis, and associated vascular responses, in addition to, or instead of aspirin.

Synergistic platelet aggregation responses have been described by many investigators for a variety of combinations of agonists (Kinlough-Rathbone & Mustard, 1986). However, the role of TXA_2 in the synergistic aggregation response between weak agonists, in particular, has not been clearly defined. Thromboxane formation has been reported to play a major role in synergism between collagen and other agonists in rabbit platelets (Kinlough-Rathbone & Mustard, 1977). It has been reported that amplification by 5-HT of collagen-induced aggregation, but not ADP-induced aggregation was affected by inhibition of cyclo-oxygenase (De Clerck *et al.*, 1982), indicating that TXA_2 was not essential in the interaction between the weak agonists 5-HT and ADP. Cameron & Ardlie (1982) showed, however, that the interaction between adrenaline and ADP operates via two distinct mechanisms, one TXA_2-dependent and the other TXA_2-independent, although in these experiments TXA_2 production in response to a combination of critical concentrations of agonists only was investigated.

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Investigating more thoroughly the mechanism by which 5-HT and adrenaline potentiate ADP-induced aggregation will determine whether additional activation pathways, apart from TXA_2 generation, should be blocked to achieve greater antithrombotic potential. If marked enhancement of aggregation by 5-HT and adrenaline does occur in the presence of aspirin, when there is no TXA_2 production, then it may be reasonable to supplement aspirin therapy with agents that block platelet 5-HT receptors and adrenoceptors.

The aim of this study was to examine platelet aggregation and TXA_2 production induced by the interaction of ADP with either 5-HT or adrenaline, by use of complete dose-response curves. We then wished to quantitate the inhibition of these responses by a combination of aspirin and the appropriate antagonist, ketanserin or yohimbine, and compare it with the effect achieved by each inhibitor alone.

Methods

Platelet preparation

Platelet donors were four healthy volunteers (2 males, 2 females, aged 24–35 years), who had denied taking any medication which may alter platelet function for at least two weeks before the start of the study. Each was studied on two independent occasions because of marked day to day variation in platelet responsiveness. Venous blood was taken into one tenth volume trisodium citrate (0.1 M, pH 7.35), platelet-rich plasma (PRP) prepared by centrifugation at 200 *g* for 11 min at room temperature and adjusted to a platelet count of 300,000 μl^{-1} with autologous platelet-poor plasma, prepared by centrifugation at 900 *g* for 20 min. The standardized PRP was stored at 37°C in an air-free syringe to maintain a constant pH, as previously described (Watts *et al.*, 1985). Aspirin (100 μM) or Tyrode buffer was incubated with PRP in the syringe.

Platelet function tests

Platelet aggregation was measured by turbidometry in two dual channel platelet aggregometers (Payton, Ontario, Canada). PRP (255 μl) was incubated in the aggregation cuvette for 3 min with either ketanserin (0.5–5 μM), yohimbine (0.09–9 μM) or Tyrode buffer (15 μl). Stock solutions of 5-HT and adrenaline were dissolved in HEPES-Tyrode buffer (pH 7.4), (Kinlough-Rathbone *et al.*, 1983) containing 1 mM ascorbic acid to prevent oxidation of the agonists (pH 7.2) (Britt *et al.*, 1988). The dilution of ascorbate containing solutions into PRP did not affect the pH of the solution and the presence of ascorbate did not affect aggregation. 5-HT (10 μM), adrenaline (subthreshold) or HEPES-Tyrode buffer containing ascorbate (15 μl) were then added 20 s before the addition of ADP (0.01–100 μM) (15 μl). (The final volume of PRP + agonists/antagonists was 300 μl). The concentration of 5-HT (10 μM) was chosen because it caused maximal potentiation and when used alone it caused less than 10% aggregation. The subthreshold concentration of adrenaline was determined as the maximum which caused less than 10% aggregation, and varied between subjects and experimental days. The extent of aggregation, 5 min after the addition of agonists, was measured as the percentage of the difference of light transmission between PRP and platelet-free plasma (prepared by centrifugation at 8,000 *g* for 2 min).

The production of TXA_2, 5 min after the addition of agonist, was measured by radioimmunoassay of the stable end-product, TXB_2, as previously described (Herd *et al.*, 1987). The detection limit of the assay was 0.2 ng ml^{-1} PRP or 0.6 pg/10^6 platelets.

Expression of results

Dose-response curves were plotted for concentration of ADP vs % aggregation (semilogarithmic plot) or TXA_2 generation (log-logarithmic plot) and EC_{50} values (concentration of ADP required to produce 50% maximal aggregation or TXA_2 generation) determined. Mean EC_{50} values were calculated from the EC_{50} for each individual experiment. Mean EC_{50} values in tables are shown as geometric mean ($\pm$ geometric s.e.mean) (to 2 significant figures), since it has been demonstrated (Fleming *et al.*, 1972) that EC_{50} and parameters derived therefrom are log-normally distributed. Maximal aggregation and TXA_2 generation responses are expressed as a geometric mean and geometric s.e.mean (g.s.e.).

Potentiation of ADP-induced responses by 5-HT and adrenaline was quantitated as a concentration ratio (CR) by dividing the EC_{50} for ADP alone by the EC_{50} for ADP in the presence of a second agonist. Concentration ratios in Tables 1 and 2 (2 significant figures) are calculated from mean EC_{50}

Table 1 The effect of aspirin $\pm$ ketanserin on aggregation induced by adenosine 5'-diphosphate (ADP) $\pm$ 5-hydroxytryptamine (5-HT, 10 μM)

	ADP EC_{50} (μM)			*ADP + 5-HT EC_{50} (μM)*		
	g.m.	− g.s.e.m.	+ g.s.e.m.	g.m.	− g.s.e.m.	+ g.s.e.m.
Buffer	1.0	0.8	1.2	0.5	0.4	0.6
Ketanserin (5 μM)	2.3	2.0	2.6	1.8	1.6	2.0
Aspirin (100 μM)	3.7	3.4	4.0	1.6	1.4	1.8
Aspirin + ketanserin	4.1	3.4	4.8	3.0	2.7	3.3

EC_{50}^1	*EC_{50}^2*	*Concentration ratio*	
ADP + buffer	ADP + 5-HT + buffer	2.1	$P<0.001$
ADP + ketanserin	ADP + buffer	2.3	$P<<0.001$
ADP + ketanserin	ADP + 5-HT + buffer	5.0	$P<<0.001$
ADP + 5-HT + ketanserin	ADP + 5-HT + buffer	3.8	$P<<0.001$
ADP + 5-HT + aspirin	ADP + 5-HT + buffer	3.4	$P<<0.001$
ADP + 5-HT + aspirin + ketanserin	ADP + 5-HT + buffer	6.5	$P<<0.001$
ADP + 5-HT + aspirin + ketanserin	ADP + 5-HT + aspirin	1.9	$P<0.005$
ADP + aspirin	ADP + 5-HT + aspirin	2.3	$P<<0.001$
ADP + aspirin + ketanserin	ADP + ketanserin	1.7	$P<0.05$
ADP + aspirin + ketanserin	ADP + aspirin	1.1	NS
ADP + aspirin	ADP + buffer	3.7	$P<<0.001$

Values shown are geometric mean (g.m.) $\pm$ geometric s.e.mean (g.s.e.m.) EC_{50} from eight experiments.
Concentration ratios (CR) are calculated from mean EC_{50}^1/EC_{50}^2. NS = not significant. A CR $>$ 1.6 is statistically significant at the 0.05 level.

Table 2 The effect of aspirin ± yohimbine (Yoh) on aggregation induced by adenosine 5'-diphosphate (ADP) ± adrenaline (Ad,0.25 μM)

	ADP EC_{50} (μM)			*ADP + Ad EC_{50} (μM)*		
	g.m.	− g.s.e.m.	+ g.s.e.m.	g.m.	− g.s.e.m.	+ g.s.e.m.
Buffer	0.8	0.7	0.9	0.2	0.1	0.3
Aspirin	2.2	2.0	2.4	1.0	0.9	1.1
Aspirin + yohimbine (0.09 μM)	–	–	–	1.5	1.4	1.6
Aspirin + yohimbine (0.9 μM)	–	–	–	1.8	1.6	2.0
Aspirin + yohimbine (9 μM)	–	–	–	2.2	2.0	2.4

EC_{50}^1	*EC_{50}^2*	*Concentration ratio*	
ADP + buffer	ADP + Ad + buffer	4.0	$P<<0.001$
ADP + Ad + aspirin	ADP + Ad + buffer	5.1	$P<<0.001$
ADP + aspirin	ADP + Ad + aspirin	2.0	$P<0.005$
ADP + Ad + aspirin + Yoh (0.09 μM)	ADP + Ad + aspirin	1.4	NS
ADP + Ad + aspirin + Yoh (0.9 μM)	ADP + Ad + aspirin	1.7	$P<0.05$
ADP + Ad + aspirin + Yoh (9.0 μM)	ADP + Ad + aspirin	2.0	$P<0.005$
ADP + Ad + aspirin + Yoh (9.0 μM)	ADP + Ad	10.0	$P<<0.001$

Values shown are geometric mean (g.m.) ± geometric s.e.mean (g.s.e.m.) EC_{50} from eight experiments.
Concentration ratios (CR) are calculated from mean EC_{50}^1/EC_{50}^2. NS = not significant. A CR > 1.6 is statistically significant at the 0.05 level.

values (3 significant figures). Increasing potentiation was quantitated as an increased CR.

Inhibition of responses was similarly quantitated as a CR, calculated from individual EC_{50} ratios for each experiment, by dividing the EC_{50} in the presence of the inhibitor by that obtained in its absence. Increased inhibition is indicated by an increased CR.

To calculate IC_{50} (the concentration to inhibit the potentiated aggregation response by 50%) for yohimbine and ketanserin (a) the maximal CR was determined, (b) the concentration of receptor antagonists to inhibit this ratio by 50% was determined from a graph of increasing CR vs concentration of receptor antagonist. The geometric mean and geometric s.e.mean (g.s.e.) for IC_{50} values were calculated from the IC_{50} for each individual experiment.

Statistical analyses

EC_{50} data in Tables 1 and 2 were analysed by analysis of variance after logarithmic transformation, and differences between mean values determined by q tests (Dixon & Massey, 1969). The *P* values obtained appear in respective tables.

Chemicals used and their respective sources were:

Aspirin (acetylsalicylic acid), yohimbine, 5-HT (5-hydroxytryptamine-creatinine-sulphate-complex), ADP (adenosine 5'-diphosphate), L-ascorbic acid (Sigma, St. Louis, MO., U.S.A.), ketanserin tartrate (Research Biochemicals Natick, MA., U.S.A.), adrenaline tartrate (David Bull Laboratories, Melbourne, Australia). All concentrations are expressed as final concentrations in PRP.

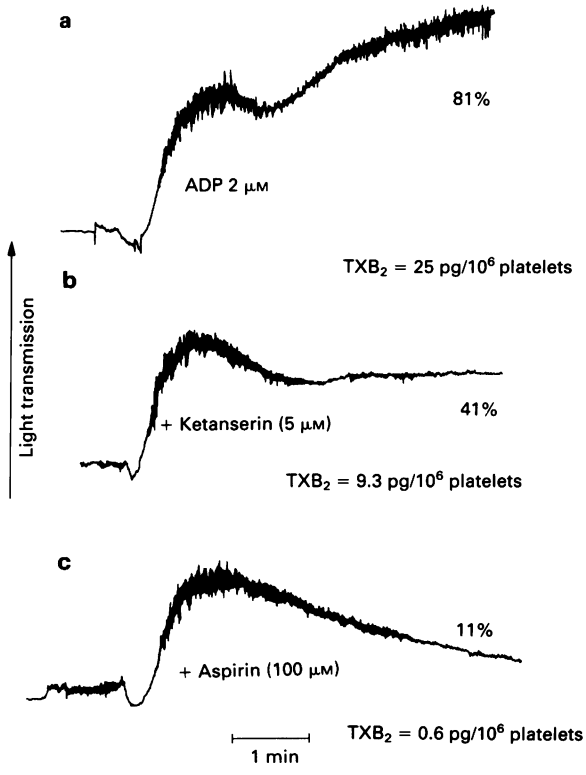

Figure 1 Platelet aggregation and thromboxane (TXB_2) production induced by adenosine 5'-diphosphate (ADP, 2 μM), after preincubation for 3 min with buffer (a), ketanserin (5 μM, b), or aspirin (100 μM, c).

Results

Responses induced by ADP

Ketanserin (5 μM) partially inhibited the second phase of aggregation induced by ADP (Figure 1), but did not affect primary aggregation. The inhibitory effect of ketanserin was observed for a range of ADP concentrations, resulting in a 2.3 fold increase in EC_{50} and a decrease in maximal aggregation response from 93 (g.s.e. 1.02) to 79 (g.s.e. 1.02)% (Figure 2a). Ketanserin also inhibited ADP-induced TXA_2 generation, resulting in a 1.8 fold increase in EC_{50} (Figure 2b). The results indicate that 5-HT released from the platelets contributes significantly to both aggregation and TXA_2 generation.

Aspirin completely inhibited the second phase of aggregation and TXA_2 generation induced by ADP (Figure 1). The inhibitory effect of aspirin on EC_{50} values for ADP-induced aggregation is shown in Tables 1 and 2. Yohimbine (9 μM) had no effect on ADP-induced aggregation or TXA_2 generation (data not shown).

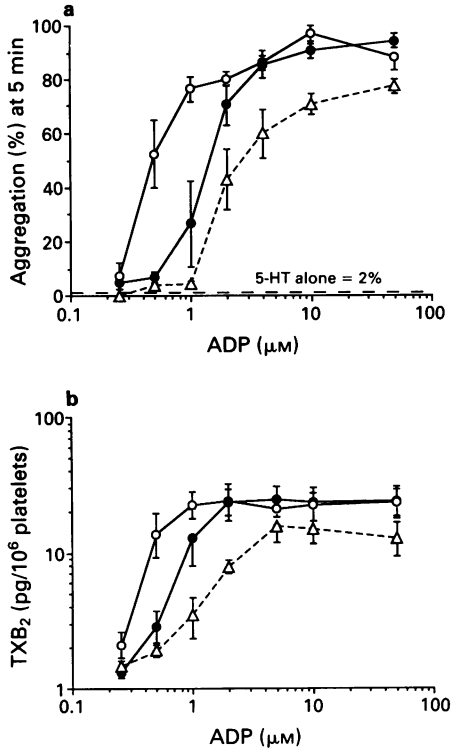

Figure 2 The effect of 5-hydroxytryptamine (5-HT) or ketanserin on platelet aggregation (a) and thromboxane generation (b) induced by adenosine 5'-diphosphate (ADP). Results are shown for 5-HT (10 μM) + ADP (○), ADP alone (●) and ADP + ketanserin (△). All points show geometric mean with s.e.mean shown by vertical bars ($n = 8$). Aggregation EC_{50} values were 0.5 μM, 1.0 μM and 2.3 μM respectively. Thromboxane EC_{50} values were 0.41 μM, 0.75 μM and 1.41 μM respectively. 5-HT (10 μM) alone induced 2 ± 1.13% aggregation (indicated by broken horizontal line) and 0.84 ± 1.63 pg thromboxane.

Responses induced by ADP and 5-HT

When examining the effect of 5-HT concentrations of 1–50 μM, potentiation of ADP-induced aggregation was found to be dose-dependent and maximal at 10 μM 5-HT. This concentration of 5-HT alone caused only 2 (g.s.e. 1.1)% platelet aggregation and TXA_2 production was negligible (0.84 (g.s.e. 1.6) pg/10^6 platelets). Prior addition of 5-HT (10 μM) caused potentiation of aggregation shown by a leftward shift of the dose-response curve to ADP (Figure 2a,b) resulting in a concentration ratio (CR) of 2.1 for ADP-induced platelet aggregation (Table 1) and 1.8 (g.s.e. 1.2) for TXA_2 generation. No increase in maximum for either response was observed.

However, as demonstrated above, endogenous (or released) 5-HT contributes to aggregation induced by ADP alone. Therefore the true aggregation induced by ADP alone is that observed in the presence of ketanserin. Hence enhancement of ADP responses by 5-HT in our study was quantitated as the combined effect of added and endogenous 5-HT, resulting in a CR of 5.0 for platelet aggregation (Figure 2a, Table 1) and 3.4 (g.s.e. 1.2) for TXA_2 generation (Figure 2b).

The effect of inhibitors on responses to ADP potentiated by 5-HT

Ketanserin The effect of added 5-HT (10 μM) on ADP-induced aggregation and TXA_2 generation was inhibited by ketanserin in a dose-dependent manner, (IC_{50} 0.53 (g.s.e. 1.2) μM). The effect of 5 μM ketanserin on the platelet aggregation responses is shown in Table 1.

Aspirin Aspirin caused inhibition of the aggregation response induced by a combination of ADP and 5-HT (CR 3.4, Table 1). However, in the presence of aspirin, ADP-induced aggregation was still potentiated by 5-HT (10 μM), with a CR of 2.3 (Table 1).

Ketanserin + aspirin In the presence of aspirin, ketanserin inhibited the potentiation by 5-HT of ADP-induced aggregation in a dose-dependent manner (IC_{50} 0.52 (g.s.e. 1.4) μM). Ketanserin (5 μM), in combination with aspirin, inhibited aggregation induced by ADP and 5-HT (CR 6.5) further than did aspirin alone (CR 3.4, Table 1).

Responses induced by ADP and adrenaline

The subthreshold concentration of adrenaline (0.25 ± 0.05 μM) caused 2 (g.s.e. 1.9)% platelet aggregation and TXA_2 production was negligible, 1.5 (g.s.e. 1.33) pg/10^6 platelets. Prior addition of this concentration of adrenaline (0.25 μM), resulted in a marked enhancement of ADP-induced aggregation. This resulted in a leftward shift of the ADP dose-response curve (Figure 3a) and a CR of 4.0 for aggregation (Table 2) and 3.5 (g.s.e. 1.2) for TXA_2 generation. No significant increase in maximal aggregation was observed. Maximal TXA_2 generation increased from 32.5 (g.s.e. 1.17) to 59.4 (g.s.e. 1.2) pg/10^6 platelets.

The effect of inhibitors on responses to ADP potentiated by adrenaline

Yohimbine Yohimbine (9 μM) inhibited the potentiation by adrenaline of ADP-induced aggregation and TXA_2 generation (results not shown).

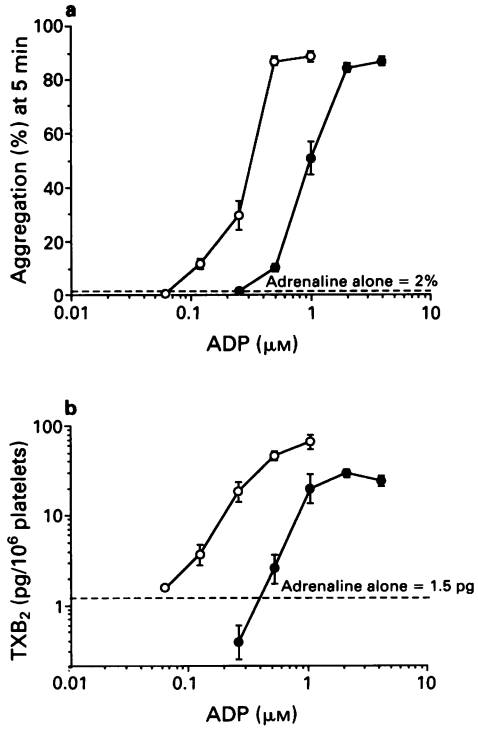

Figure 3 The effect of adrenaline on platelet aggregation (a) and thromboxane generation (b) induced by adenosine 5'-diphosphate (ADP). Results are shown for adrenaline (0.25 μM) + ADP (○) and ADP alone (●). All points show geometric mean with s.e.mean shown by vertical bars ($n = 8$). Aggregation EC_{50} values were 0.2 μM and 0.8 μM respectively. Thromboxane EC_{50} values were 0.24 μM and 0.82 μM respectively. Adrenaline (0.25 μM) alone induced 2 ± 1.9% aggregation and 1.51 ± 1.3 pg thromboxane (indicated by broken horizontal line).

Aspirin Aggregation induced by adrenaline and ADP in combination, was reduced by aspirin (CR 5.1, Table 2). However, in the presence of aspirin, ADP-induced aggregation was still potentiated by adrenaline (0.25 μM) with a CR of 2.0 (Table 2). Increasing potentiation occurred with escalating concentrations of adrenaline, with maximal potentiation achieved with 10 μM, which resulted in a CR of 7.5 (g.s.e. 1.16), ($n = 4$) (results not shown).

Yohimbine and aspirin Yohimbine, in combination with aspirin, caused further inhibition of the aggregation induced by ADP and adrenaline (CR 10.0), than did aspirin alone (CR 5.1, Table 2). In the presence of aspirin, yohimbine inhibited potentiation by adrenaline of ADP-induced aggregation in a dose-dependent manner (Table 2) (IC_{50} 0.22 (g.s.e. 1.36) μM). Yohimbine (9 μM) completely inhibited the effect of added adrenaline, and the EC_{50} for aggregation was then 2.2 μM, the same as for ADP alone in the presence of aspirin (Table 2).

Discussion

We have used complete dose-response curves to show that there was a synergistic interaction for aggregation and thromboxane generation achieved by 5-HT and adrenaline in combination with ADP. To identify true synergism it was necessary to demonstrate a parallel shift to the left of the dose-response curve to one agonist in the presence of a threshold concentration of the other. If an interaction between two agonists is additive rather than synergistic, the dose-response curve for the combined agonists will not be parallel to the dose-response curve for one agonist alone (Draskóczy & Trendelenburg, 1968; Asano & Hidaka, 1980). A superadditive response to a combination of threshold doses of two agonists, as reported in the great majoritiy of previous studies (Kinlough-Rathbone & Mustard, 1986), is therefore not sufficient if true synergism is to be demonstrated.

We have shown that the aggregation response induced by either 5-HT or adrenaline in combination with ADP was synergistic, both in the presence and absence of aspirin. Several studies have demonstrated synergism between platelet agonists in the absence of a cyclo-oxygenase inhibitor (Mills & Roberts, 1967; Michal & Motamed, 1976; Osmani *et al.*, 1983; Steen & Holmsen, 1985). In the presence of a cyclo-oxygenase inhibitor, synergism between ADP and adrenaline or 5-HT was observed only when aggregation was measured by light transmission, but not by decrease in numbers of single platelets in suspension (Thompson *et al.*, 1986). Ours is the first study in which synergism has been investigated both in the presence and absence of aspirin. This has allowed us to assess the contribution of TXA_2 to the synergistic aggregation response.

To quantitate the effects of 5-HT and adrenaline on the ADP response it was necessary first to assess the effects of released (endogenous) 5-HT and adrenaline on the response to ADP alone. When specific inhibitors of these substances were added to platelets prior to ADP, yohimbine was without effect, but ketanserin had an inhibitory effect on the second phase of ADP-induced platelet aggregation. We can conclude therefore, in agreement with previous investigators, that endogenous 5-HT contributes significantly to the second phase of the ADP-induced aggregation response (Bevan & Heptinstall, 1983; De Clerck & Xhonneux, 1985; Heptinstall *et al.*, 1988). In this study we have extended this observation, which in the earlier studies examined aggregation induced by critical agonist concentrations only, to show that ketanserin (5 μM) inhibits both aggregation and TXA_2 generation induced by a range of concentrations of ADP. This finding shows that released 5-HT stimulates the production of TXA_2. Although ketanserin is a selective 5-HT_2 receptor antagonist, it has a moderate α_1-adrenoceptor antagonist effect (Leysen *et al.*, 1981). Kerry & Scrutton (1985) have demonstrated that platelet α_1-adrenoceptor density on platelets is negligible. There has been no reported evidence of ketanserin binding to purinoceptors, which is consistent with the fact that no decrease in ADP-induced primary aggregation was seen. The effect of ketanserin on secondary aggregation induced by ADP is therefore due to blockade of 5-HT_2 receptors and inhibition of positive feedback aggregation and thromboxane generating responses caused by released endogenous 5-HT.

When 5-HT was added prior to ADP, there was a parallel shift to the left for both aggregation and thromboxane formation, confirming previous aggregation results in rabbit platelets (Michal & Motamed, 1976). The degree of shift was more marked when endogenous 5-HT was taken into account. If ADP-induced aggregation and thromboxane formation in the presence of ketanserin are considered the true baseline ADP responses, then an increase in maximum as well as a leftward shift by the addition of 5-HT was observed. In the presence of aspirin, 5-HT retained its ability to enhance ADP-induced aggregation. This effect was less (CR 2.3) than in the absence of aspirin (CR 5.0). Therefore the effect of 5-HT on ADP-induced aggregation is enhanced by, but does not depend on, TXA_2 production. De Clerck *et al.* (1982) found that the effect of 5-HT on ADP-induced aggregation was of the same magnitude whether or not TXA_2 was inhibited, but it is not clear whether the effects of endogenous 5-HT were taken into account.

Experiments with adrenaline also showed a parallel shift to the left of the ADP dose-response curves for both aggregation and TXA_2 generation, confirming previous aggregation data (Mills & Roberts, 1967). The CR of 4.0 achieved for aggregation was reduced to 2.0 in the presence of aspirin, indicating that some of the potentiation previously observed was TXA_2-dependent. However in the presence of aspirin, a sub-threshold dose of adrenaline was still able to potentiate the aggregation response induced by ADP (CR 2.0). Furthermore, in the presence of aspirin, higher doses of adrenaline caused marked potentiation (CR 7.5) of ADP-induced aggregation by a TXA_2-independent mechanism. This extends preliminary observations by Cameron & Ardlie (1982) that critical concentrations of adrenaline and ADP interact to cause a greater than additive response in the presence or absence of aspirin, and Rao *et al.* (1980) who showed that in aspirin-treated platelets, adrenaline (2.5 μM) caused the previously monophasic response induced by ADP (2 μM) to become irreversible. We have quantitated both TXA_2-dependent and independent potentiation by adrenaline of ADP-induced aggregation, and demonstrated that adrenaline and ADP interact in a truly synergistic manner. The intracellular pathways involved remain to be identified.

After examining the nature of the potentiation mechanisms of adrenaline and 5-HT, we considered the ability of the specific 5-HT and adrenaline antagonists, ketanserin and yohimbine, alone and in combination with aspirin, to inhibit the synergistic responses between dual agonists. While previous studies have shown yohimbine and ketanserin effectively inhibit potentiation of platelet responses by adrenaline and 5-HT, to our knowledge the combination of these agents with aspirin has not been reported.

The combination of ketanserin (0.5–5 μM) and aspirin inhibited the platelet aggregation response induced by 5-HT and ADP further than either agent alone. The IC_{50} of ketanserin obtained in our study (0.52 μM) was similar to that obtained in several other *in vitro* studies (Arnout *et al.*, 1985; Schächter *et al.*, 1985), and is consistent with levels shown to be effective *in vivo* (The PACK trial group, 1989). Much lower IC_{50} levels were obtained in some other studies in PRP (16.6 nM, De Clerck *et al.*, 1982; 23 nM, De Clerck *et al.*, 1984; 33 nM, Glusa & Markwardt, 1984). The difference in IC_{50} between these last studies and our study cannot be explained by the 95% protein binding of ketanserin in plasma (Persson *et al.*, 1991), or by uncontrolled platelet storage pH previously stated as the reason for a lower

potency of ketanserin (De Clerck et al., 1985). Our storage conditions, of platelet-rich plasma in an air-free syringe at 37°C, ensure that pH is maintained at 7.3–7.4 throughout the experiment (Watts et al., 1985).

Yohimbine inhibited the enhancement by adrenaline of ADP-induced platelet aggregation, indicating that this enhancement is mediated by α_2-adrenoceptors, as shown previously by others (Grant & Scrutton, 1979; Lanza & Cazenave, 1985). Enhancement by adrenaline of the aggregation response was inhibited to a certain degree in the presence of aspirin alone, but when yohimbine was also present the remaining potentiation was maximally inhibited. In previous studies, not however in the presence of aspirin, yohimbine has been shown to inhibit effectively potentiation by adrenaline of ADP-induced aggregation in PRP (Grant & Scrutton, 1979), with IC_{50} (0.5–1.2 μM) values similar to those obtained in our study (0.22 μM). This is similar to the plasma yohimbine concentration (0.19 μM) achieved *in vivo* after a 10 mg oral dose (Owen et al., 1987), or an 8 mg oral dose (Berlin et al., 1991) which was shown to inhibit effectively platelet aggregation induced by adrenaline (5 μM). Our results are in agreement with those of Rao et al. (1981) who showed that yohimbine (5–10 μM) blocked potentiation by adrenaline of sodium arachidonate-induced aggregation in PRP from donors who had taken aspirin (80 mg, single dose). In washed platelets the IC_{50} (19.5 nM, Lanza & Cazenave, 1985) for the inhibition of potentiation of ADP (1 μM)-induced aggregation by adrenaline (0.5 μM), was lower than in our study in PRP, suggesting that plasma protein binding of the drug may be occurring in PRP.

The maximal potentiating concentration of adrenaline (10 μM) and 5-HT (10 μM) produced a CR of 7.5 and 2.3 respectively, in the presence of aspirin. Therefore adrenaline is more potent for potentiating ADP-induced aggregation in this experimental system. Conversely, the platelet adrenaline content (23 pmol/2.86 × 10^8 platelets) is approximately three orders of magnitude lower than 5-HT (1 μmol/2.86 × 10^8 platelets) (Da Prada & Picotti, 1979). The concentrations of 5-HT and ADP used in this study are within the plasma concentration range that can be achieved after maximal platelet secretion (Meyers et al., 1982). The concentration of adrenaline is approximately 100 times greater than circulating *in vivo* catecholamine concentrations (Ardlie et al., 1984) although this does not take into account conditions of stress or the higher local agonist concentrations which occur in the platelet microenvironment at the site of a thrombus.

Yohimbine or ketanserin have the potential to be used in combination with aspirin as antiplatelet agents *in vivo*. Apart from their action as mediators of platelet aggregation, 5-HT and TXA_2 are also potent vasoconstrictors and inducers of vasospasm, which in conjunction with intracoronary platelet aggregation, may play a crucial role in the genesis of unstable angina and myocardial infarction (Fitzgerald et al., 1986; Mehta, 1990). Ketanserin, in combination with thromboxane receptor antagonists, but not singly, has been reported to inhibit coronary artery reocclusion following thrombolytic therapy with tissue plasminogen activator in canines (Golino et al., 1988), and similarly aspirin has been successful in this regard after streptokinase therapy in man (ISIS-2 collaborative group, 1988). The combination of aspirin and ketanserin to inhibit potentiation by 5-HT of platelet and blood vessel responses may therefore prove to be more successful in the primary and secondary prevention of myocardial ischaemia than aspirin alone.

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