Br. J. Pharmac. (1986), **88**, 931–936

Prostaglandin D_2 interacts at thromboxane receptor-sites on guinea-pig platelets

S. Hamid-Bloomfield & B.J.R. Whittle

Department of Mediator Pharmacology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The anti-aggregatory prostanoid, prostaglandin D_2 (PGD_2) does not completely inhibit ADP-induced aggregation of guinea-pig platelets and thus produces a bell-shaped dose-inhibition curve. The nature of this bell-shaped curve has now been investigated in guinea-pig platelet-rich plasma.

2 Two selective thromboxane receptor antagonists, 13-aza-prostanoic acid (13-AZA; 16–64.4 μM) and BM 13.177 (5.9–29.8 μM), converted PGD_2 to a full inhibitor of aggregation in a dose-related manner.

3 The putative platelet PGD_2 receptor antagonist, N-0164 (75 μM) also converted PGD_2 to a full inhibitor of platelet aggregation. In contrast to 13-AZA and BM 13.177, higher concentrations of N-0164 (380 and 760 μM) caused a dose-related rightward shift of the PGD_2 dose-inhibition curve.

4 The thromboxane receptor antagonism of N-0164 was confirmed in studies in which the dose-aggregation curve to U-46619, a thromboxane mimetic, was competitively antagonized with a pA_2 value of 4.67 and a slope of 1.13, comparable to that of 13-AZA.

5 The results show that N-0164 acts as both a platelet PGD_2 and thromboxane-receptor antagonist in both human and guinea-pig platelet-rich plasma.

6 The results further indicate that PGD_2 can interact at thromboxane receptors in guinea-pig platelets.

Introduction

Prostaglandin D_2 (PGD_2) has potent anti-aggregatory activity on platelets of several species (Whittle *et al.*, 1978). As with the other prostanoid inhibitors of platelet aggregation, prostaglandin E_1 (PGE_1) and prostacyclin (PGI_2), PGD_2 elevates platelet adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels which supports the concept of a similar intracellular mechanism of action for these prostanoids (Tateson *et al.*, 1977; Gorman *et al.*, 1977; Whittle *et al.*, 1978). However, the platelet receptors mediating the responses to PGD_2 appear distinct from those of PGE_1 and PGI_2. Thus, the experimental compound N-0164 in appropriate concentrations selectively antagonized the anti-aggregatory actions of PGD_2 with no effect on those of PGE_1 and PGI_2 on human platelets (MacIntyre & Gordon, 1977; Whittle *et al.*, 1978).

Our previous studies in guinea-pig platelet-rich plasma (PRP) have shown that PGD_2 produces a bell-shaped dose-anti-aggregatory relationship, with complete inhibition of platelet aggregation not being achieved (Hamid & Whittle, 1985). An earlier study has shown that the thromboxane receptor antagonist EP045 partially blocks the contractile actions of PGD_2 on the guinea-pig trachea which thus could be indicative of an interaction of PGD_2 at thromboxane-sensitive contractile sites (Jones *et al.*, 1982). In the present study, we have therefore investigated whether the interaction of PGD_2 at the thromboxane receptor sites in guinea-pig platelets could explain the nature of the dose-response relationship using two selective thromboxane antagonists, 13-aza-prostanoic acid (13-AZA; Venton *et al.*, 1979) and BM 13.177 (Patscheke *et al.*, 1984). In addition, we have compared the activity of N-0164 as an antagonist of these PGD_2 responses in guinea-pig PRP.

A preliminary account of this work has been presented to the British Pharmacological Society (Hamid & Whittle, 1986).

Methods

The method was as described previously by Hamid & Whittle (1985). Male Halls guinea-pigs (350–450 g)

were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and 15 ml of blood was collected from the abdominal aorta into plastic tubes containing trisodium citrate (final concentration 0.315%). The blood was centrifuged (1000 *g*) for 2 min to obtain platelet-rich plasma (PRP) which was removed and stored at room temperature. The platelet count in the PRP, determined using a Coulter Counter (model ZF), was $2.8-3.5 \times 10^8$ ml^{-1}.

Platelet aggregation was measured in a Payton dual channel aggregation module connected to a 'W + W' recorder 1200. Samples (0.5 ml) of PRP were incubated for 1 min at 37°C, stirred at 900 r.p.m. with or without the prostaglandin under investigation, prior to addition of submaximal doses of adenosine diphosphate (ADP, 2–4 μM) sufficient to just cause a non-reversible control aggregation.

Dose-inhibition curves were constructed for PGD_2, prostacyclin (PGI_2) and BW245C and the IC_{50} was calculated as the concentration required to reduce the aggregation to 50% of its control amplitude.

To study the interaction of PGD_2 at thromboxane receptors, the PRP was preincubated (1 min at 37°C) with the two selective thromboxane antagonists (13-AZA and BM 13.177) before adding the appropriate concentration of PGD_2 for a further 1 min incubation, before the addition of ADP.

The activity of N-0164 as an antagonist of the PGD_2, BW245C and PGI_2 anti-aggregating responses was studied in a comparable fashion. In further studies, to investigate whether N-0164 was acting as a thromboxane receptor antagonist, guinea-pig PRP was preincubated (1 min) with N-0164 (19–760 μM) before the addition of the epoxy-methano endoperoxide analogue U-46619 (0.11–1.14 μM), a thromboxane mimetic (Coleman *et al.*, 1981). Likewise, the actions of N-0164 (38–760 μM) on U-46619 (0.11–2.8 μM)-induced aggregation of human PRP, prepared as described previously (Whittle *et al.*, 1978), were investigated.

Drugs

Prostacyclin as the sodium salt (Wellcome Foundation) was freshly dissolved in 1 M Tris buffer (pH 9.6 at 4°C) immediately prior to use. Prostaglandin D_2; 11α, 9α epoxy-methano PGH_2, U-46619 (from the Upjohn Company, Kalamazoo, U.S.A.) and the hydantoin prostaglandin 5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl hydantoin (BW245C, synthesized in the Dept Therapeutic Chemistry, Wellcome Research Laboratories) were stored in ethanol (10 mg ml^{-1}; 4°C) and diluted with 50 mM Tris buffer (pH 7.5 at 4°C) when required. Adenosine diphosphate (Sigma Chemical Co.) was dissolved in distilled water when required and kept on ice.

The thromboxane antagonists, 13-aza-prostanoic acid (13-AZA) and 4-[2-(benzyenesulphonamide)-ethyl]-phenoxyacetic acid (BM 13.177) were synthesized in the Dept Therapeutic Chemistry, Wellcome Research Laboratories by Dr W. Jackson. These compounds were stored in ethanol (10 mg ml^{-1}; 4°C) and diluted with 50 mM Tris buffer (pH 7.5 at 4°C) when required. Compound N-0164 (sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl]phenyl phosphonate, obtained from Nelson Research and Development Company, California) was freshly dissolved in distilled water (10 mg ml^{-1} at 37°C).

Statistical analysis

Results are expressed as mean (± s.e.mean), where *n* is the number of values. The difference between groups was evaluated using Student's *t* test for unpaired data, where $P < 0.05$ was taken as significant. Compliance to simple competitive antagonism was tested by comparison of dose-ratios of agonists, obtained in the presence of antagonist, to the Schild equation where the abscissal intercept represents the empirical measure of potency, pA_2 (Arunlakshana & Schild, 1959). Linear line regression analysis with 95% confidence limits was carried out on these data. The slopes and asymptotes of the dose-response curves were analysed using the *F* test of equal curves.

Results

Effects of the thromboxane-receptor antagonists

Pre-incubation of guinea-pig PRP with PGD_2 produced a bell-shaped dose-anti-aggregatory relationship, with complete inhibition of platelet aggregation induced by ADP not being achieved (Figure 1), confirming previous findings (Hamid & Whittle, 1985). The effects of two selective thromboxane receptor antagonists 13-AZA and BM 13.177, on this bell-shaped dose-response relationship with PGD_2 were investigated. Both 13-AZA (16–64.4 μM) and BM 13.177 (5.9–29.8 μM) significantly converted, in a dose-related manner, the responses of PGD_2 to a full inhibition of aggregation (Figures 1 and 2). Higher concentrations of 13-AZA (129 μM) and BM 13.177 (60 μM) did not further shift the PGD_2 curves. BM 13.177 was more potent in converting PGD_2 to a full inhibitor of ADP-induced aggregation than 13-AZA.

Effects of N-0164

N-0164 at a low concentration (75 μM) converted the PGD_2 bell-shaped curve to a sigmoid dose-inhibition curve, exposing it as a full inhibitor of ADP-induced aggregation (Figure 3a). In contrast to 13-AZA and

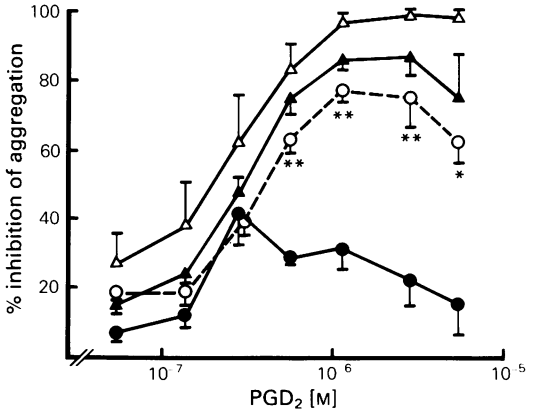

Figure 1 Effect of the thromboxane antagonist 13-aza-prostanoic acid (13-AZA) on the inhibition of ADP-induced aggregation of guinea-pig platelets by prostaglandin D_2 (PGD_2). Control PGD_2 curve (●, $n = 3$), PGD_2 in the presence of 16 μM 13-AZA (○, $n = 3$), PGD_2 + 32 μM 13-AZA (▲, $n = 3$) and PGD_2 + 64 μM 13-AZA (△, $n = 3$). Results, expressed as mean (with vertical lines indicating s.e.mean) % inhibition of platelet aggregation, where statistical differences from control PGD_2 curve are shown as $*P < 0.05$; $**P < 0.01$.

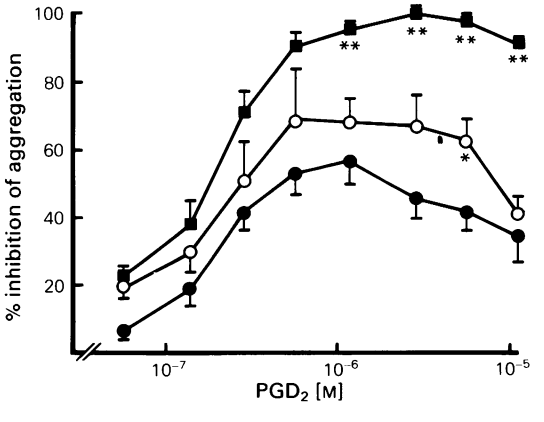

Figure 2 Effect of the thromboxane antagonist BM 13.177 on the inhibition of ADP-induced aggregation of guinea-pig platelets by prostaglandin D_2 (PGD_2). Control PGD_2 curve (●, $n = 8$), PGD_2 + 6 μM BM 13.177 (○, $n = 4$), PGD_2 + 30 μM BM 13.177 (■, $n = 6$). Results, expressed as mean (with vertical lines indicating s.e.mean) % inhibition of platelet aggregation. $*P < 0.05$; $**P < 0.001$.

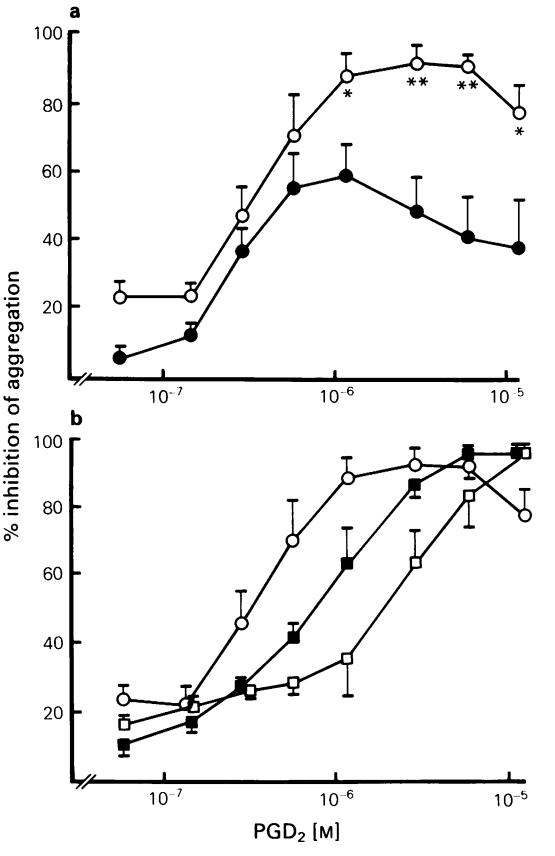

Figure 3 Effect of N-0164 on the inhibition of ADP-induced aggregation of guinea-pig platelets by prostaglandin D_2 (PGD_2). (a) Shows responses to PGD_2 (●, $n = 8$), PGD_2 + 75 μM N-0164 (○, $n = 3$), while (b) shows effect of PGD_2 + 75 μM N-0164 (○, $n = 3$). PGD_2 + 380 μM N-0164 (■, $n = 4$) and PGD_2 + 760 μM N-0164 (□, $n = 4$). Results shown are means (with vertical lines indicating s.e.mean). $*P < 0.05$; $**P < 0.001$.

BM 13.177, increased concentrations of N-0164 (380 and 760 μM) caused a dose-related rightward shift of PGD_2 dose-inhibitory response curves, with the IC_{50} value (0.29 ± 0.02 μM, $n = 4$) being increased to 0.9 ± 0.18 and 2 ± 0.6 μM, respectively ($P < 0.001$; Figure 3b). These concentrations of N-0164 themselves had no consistent effect on the aggregation induced by ADP. Due to the complex nature of the PGD_2 dose-inhibition curve through its apparent interaction at thromboxane receptors, it was not possible to carry out a true Schild plot regression analysis to obtain a pA_2 value for N-0164 as an antagonist of PGD_2. However, a calculated pA_2 value of 3.6 for this action was derived. Furthermore, in the

Table 1 Estimates of N-0164 as a prostaglandin D_2 (PGD_2) antagonist and of N-0164 and 13-aza-prostanoic acid (13-AZA) as thromboxane receptor antagonists (using the epoxy-methano endoperoxide analogue U-46619 as the thromboxane agonist) in the guinea-pig platelet-rich plasma

Agonist	*Antagonist*	*pA_2*	*Confidence limits (95%)*	n	*Slope*	*Confidence limits (95%)*
PGD_2	N-0164	3.6 (estimated)	—	(4)		—
PGD_2 in the presence of BM 13.177	N-0164	3.64 (estimated)	—	(3)		—
U-46619	N-0164	4.67	4.87–4.52	(3)	1.13	0.93–1.32
U-46619	13-AZA	4.83	4.92–4.75	(3)	1.36	1.15–1.57

The results are expressed as pA_2 values and Schild-plot slope values (with 95% confidence limits) from (n) experiments.

presence of BM 13.177 (30 μM) in a concentration which converted PGD_2 to a full inhibitor of aggregation, N-0164 (760 μM) produced a rightward shift of the PGD_2 dose-inhibitory response curve with the IC_{50} value (0.2 ± 0.02 μM, $n = 6$) being increased to 0.9 ± 0.16 μM ($n = 4$, $P < 0.001$). Under these conditions, the pA_2 value for N-0164 as a PGD_2 antagonist was calculated to be 3.64 (Table 1).

Analysis using the F test of equal curves showed that the dose-response curve to PGI_2 was not significantly different from that in the presence of N-0164 (760 μM, $P > 0.05$); only the lower asymptote was found to be different from the control curve (Figure 4). In contrast, N-0164 (760 μM) not only changed the lower asymptote of the BW245C dose-response curve, but also significantly changed the slope of the curve ($P < 0.0001$) and caused a rightward shift as shown in Figure 4. There was no significant difference in the

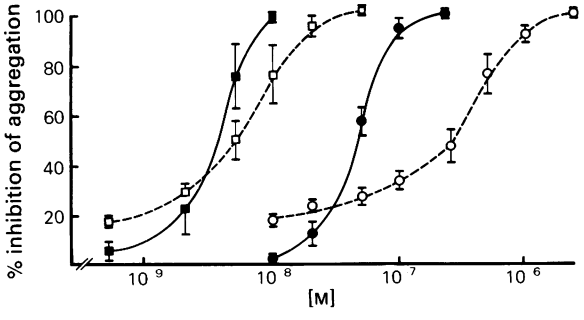

Figure 4 Effect of N-0164 on prostacyclin (PGI_2)- or BW245C-induced inhibition of guinea-pig platelet aggregation. Control PGI_2 (■, $n = 4$), in the presence of 760 μM N-0164 (□, $n = 4$); control BW245C (●, $n = 4$) curve in the presence of N-0164 (760 μM, ○, $n = 4$). Results shown are means (with vertical lines indicating s.e.mean).

IC_{50} for PGI_2 in the presence of 760 μM N-0164 (3.5 ± 0.8 nM and 6.4 ± 1.2 nM respectively), whereas the IC_{50} value for BW245C was increased from 46 ± 4 nM to 277 ± 55 nM ($P < 0.01$).

Thromboxane-receptor antagonism by N-0164

To investigate further whether N-0164 was acting as a thromboxane receptor antagonist, its effects on aggregation induced by U-46619, a thromboxane mimetic (Coleman *et al.*, 1981), were evaluated. N-0164 (38–760 μM) shifted the U-46619 dose-aggregation curve to the right in a concentration-related manner, with a pA_2 value of 4.67 in guinea-pig PRP (Table 1). This value was comparable to that obtained with the selective thromboxane receptor antagonist 13-AZA in guinea-pig platelets (Table 1). Likewise, in three experiments with human PRP, N-0164 induced a rightward shift of the dose-aggregation curve to U-46619, with a pA_2 of 4.09 (4.28–3.96) and a slope of 1.05 (0.76–1.34).

Discussion

We have previously shown that PGD_2 produced a bell-shaped dose-inhibition curve in guinea-pig PRP, with complete inhibition of ADP-induced platelet aggregation not being achieved (Hamid & Whittle, 1985). We proposed several possibilities to explain this bell-shaped curve, one of them being that at high concentrations PGD_2 might be interacting with other stimulatory and pro-aggregatory receptors on guinea-pig platelets, such as those for thromboxane A_2. In an earlier study, Jones *et al.* (1982) suggested that PGD_2 might be interacting at thromboxane-sensitive contractile sites since EP045, a thromboxane receptor antagonist, partially blocked the contractile actions of PGD_2 on the guinea-pig trachea. It has further been

suggested that the constriction of a helical strip of the dog cerebral artery by PGD_2 could be mediated via $PGF_{2\alpha}$ and/or thromboxane receptors (Narumiya & Toda, 1985).

Using two selective thromboxane antagonists of divergent structures, 13-AZA and BM 13.177, the results of the present study support the suggestion that at high concentrations PGD_2 can interact at platelet thromboxane receptors. Both, 13-AZA and BM 13.177, converted PGD_2 to a full inhibitor of ADP-induced aggregation of guinea-pig platelets in a dose-related manner, BM 13.177 being the more potent. Hence it appears that the secondary phase of the bell-shaped curve to PGD_2 at high concentrations reflects stimulation of thromboxane receptors. In the human PRP, however, PGD_2 acts as a full potent inhibitor of ADP-induced platelet aggregation and probably would require much higher concentrations to stimulate the thromboxane receptor sites. Although the hydantoin BW245C is considered to interact with platelet PGD_2 receptors (Whittle *et al.*, 1983; Town *et al.*, 1983; Hamid & Whittle, 1985) 13-AZA did not affect its full dose-inhibition curve, indicating that this compound does not interact at platelet thromboxane receptors.

Previous studies on human platelets have shown that N-0164 acts as a selective antagonist of the anti-aggregatory actions of PGD_2 (MacIntyre & Gordon, 1977; Whittle *et al.*, 1978). In the present study in guinea-pig PRP, N-0164 at low concentrations (75 μM), like the thromboxane antagonists 13-AZA and BM 13.177, converted the PGD_2 bell-shaped curve to a sigmoid dose-inhibition curve, exposing it as a full inhibitor of aggregation. In contrast to the thromboxane receptor antagonists, however, increased concentrations of N-0164 (380 and 760 μM) caused a dose-related rightward shift of the PGD_2 dose-inhibition curves, indicating antagonism at the PGD_2 receptor site. The apparent pA_2 value of N-0164 as a PGD_2 antagonist was very similar when calculated in the absence and presence of BM 13.177. Furthermore, although N-0164 did not significantly shift the dose-inhibition curve for PGI_2, it did cause a rightward shift of the curve for BW245C. These findings are consistent with the previous studies that PGD_2 acts at platelet receptor sites distinct from those of prostacyclin, and that BW245C interacts with PGD_2 (but not thromboxane)-sensitive sites (Whittle *et al.*, 1978, 1983; Seigl *et al.*, 1979; Schafer *et al.*, 1979; Miller & Gorman, 1979.

Since low concentrations of N-0164 converted the PGD_2 bell-shaped curve to that of a full inhibitor of aggregation in a manner similar to those of the two thromboxane antagonists, it was of interest to determine whether N-0164 could act as an antagonist against platelet aggregation induced by U-46619, a thromboxane mimetic (Coleman *et al.*, 1981). N-0164 acted as a thromboxane antagonist with a pA_2 value comparable to that of the thromboxane antagonist 13-AZA in both guinea-pig and human platelets. Although N-0164 has been found to be a PGD_2 antagonist in the platelet (MacIntyre & Gordon, 1977; Whittle *et al.*, 1978), the present study suggests that it is more potent as a thromboxane receptor antagonist in guinea-pig platelets.

These results thus show that there is a fine balance and interaction between PGD_2 and thromboxane receptor-sites. In guinea-pig platelets, PGD_2 appears to have at least two actions, those at PGD_2 receptor-sites and those at thromboxane receptor sites. The close relationship between platelet PGD_2 receptors and thromboxane receptors is also reflected in the finding that N-0164, the putative PGD_2 antagonist, acts as a thromboxane antagonist. This PGD_2-thromboxane interaction has also been observed in recent studies with the experimental compound AH 6809, which antagonized both PGD_2- and U-46619-induced responses in human PRP with pA_2 values of 5.4 and 4.4, respectively (Keery & Lumley, 1985). Clearly, however, not all thromboxane antagonists non-specifically antagonize PGD_2 receptors (Bennett & Sanger, 1982; Armstrong *et al.*, 1985) as confirmed in the present study. The full characterization of both *in vitro* and *in vivo* responses to PGD_2 in various tissues will necessarily require the use of both specific thromboxane- and PGD_2-receptor antagonists.

References

ARMSTRONG, R.A., JONES, R.L. PEESAPATI, V., WILL, S.G. & WILSON, N.H. (1985). Competitive antagonism at thromboxane receptors in human platelets. *Br. J. Pharmac.*, **84**, 595–607.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac. Chemother.*, **14**, 48–58.

BENNETT, A. & SANGER, G.J. (1982). Pinane thromboxane A_2 analogues are non-selective prostanoid antagonists in rat and human stomach muscle. *Br. J. Pharmac.*, **77**, 591–596.

COLEMAN, R.A., HUMPHREY, P.P.A., KENNEDY, I., LEVY, G.P. & LUMLEY, P. (1981). Comparison of the actions of U-46619, a prostaglandin H_2-analogue, with those of prostaglandin H_2 and thromboxane A_2 on some isolated smooth muscle preparations. *Br. J. Pharmac.*, **73**, 773–778.

GORMAN, R.R., BUNTING, S. & MILLER, O.V. (1977). Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins*, **13**, 377–388.

HAMID, S. & WHITTLE, B.J.R. (1985). Interaction of prostaglandin D_2 with prostacyclin, carbacyclin and the

hydantoin prostaglandin, BW245C, in guinea-pig platelets. *Br. J. Pharmac.*, **85,** 285–290.

HAMID, S. & WHITTLE, B.J.R. (1986). Antagonism of thromboxane and prostaglandin D_2 responses by N-0164 in guinea-pig platelets. *Br. J. Pharmac. Proc. Suppl.*, **87,** 110P.

JONES, R.L., PEESAPATI, V. & WILSON, N.H. (1982). Antagonism of the thromboxane-sensitive contractile systems of the rabbit aorta, dog saphenous vein and guinea-pig trachea. *Br. J. Pharmac.*, **76,** 423–438.

KEERY, R.J. & LUMLEY, P. (1985). AH 6809, a selective antagonist at the human platelet DP-receptor? *Br. J. Pharmac. Proc. Suppl.*, **85,** 286P.

MACINTYRE, D.E. & GORDON, J.L. (1977). Discrimination between platelet prostaglandin receptors with a specific antagonist of bisenoic prostaglandins. *Thromb. Res.*, **11,** 705–713.

MILLER, O.V. & GORMAN, R.R. (1979). Evidence for distinct prostaglandin I_2 and D_2 receptors in human platelets. *J. Pharmac. exp. Ther.*, **210,** 134–140.

NARUMIYA, S. & TODA, N. (1985). Different responsiveness of prostaglandin D_2-sensitive systems to prostaglandin D_2 and its analogues. *Br. J. Pharmac.*, **85,** 367–375.

PATSCHEKE, H. & STEGMEIER, K. (1984). Investigations on a selective non-prostanoic thromboxane antagonist, BM 13.177, in human platelets. *Thromb. Res.*, **33,** 277–288.

SCHAFER, A.I., COOPER, B., O'HARA, D. & HANDIN, R.I. (1979). Identification of platelet receptors for prostaglandin I_2 and D_2. *J. biol. Chem.*, **254,** 2914–2917.

SIEGL, A.M., SMITH, J.B. & SILVER, M.J. (1979). Selective binding site for [^{3}H]-prostacyclin on platelets. *J. clin. Invest.*, **63,** 215–220.

TATESON, J.E., MONCADA, S. & VANE, J.R. (1977). Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins*, **13,** 389–397.

TOWN, M-H., CASALS-STENZEL, J. & SCHILLINGER, E. (1983). Pharmacological and cardiovascular properties of a hydantoin derivative, BW245C, with high affinity and selectivity for PGD_2 receptors. *Prostaglandins*, **25,** 13–28.

VENTON, D.L., ENKE, S.E. & LE BRETON, G.C. (1979). Azaprostanoic acid derivatives: Inhibition of arachidonic acid induced platelet aggregation. *J. med. Chem.*, **22,** 824–830.

WHITTLE, B.J.R., MONCADA, S., MULLANE, K. & VANE, J.R. (1983). Platelet and cardiovascular activity of the hydantoin BW245C, a potent prostaglandin analogue. *Prostaglandins*, **25,** 205–223.

WHITTLE, B.J.R., MONCADA, S. & VANE, J.R. (1978). Comparison of the effects of prostacyclin (PGI_2), prostaglandin E_1 and D_2 on platelet aggregation in different species. *Prostaglandins*, **16,** 373–388.

(Received February 25, 1986.
Revised April 17, 1986.
Accepted April 21, 1986.)