Br. J. Pharmac. (1985), **85**, 285–290

Interaction of prostaglandin D_2 with prostacyclin, carbacyclin and the hydantoin prostaglandin, BW245C, in guinea-pig platelets

S. Hamid & B.J.R. Whittle

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

1 The anti-aggregating actions of prostaglandin D_2 (PGD_2) have been compared to prostacyclin (PGI_2), its stable analogue carbacyclin and a hydantoin prostaglandin, BW245C, in guinea-pig platelets *in vitro*.

2 PGI_2, carbacyclin and BW245C were potent inhibitors of ADP-induced aggregation in guinea-pig platelets, with ID_{50} values comparable to those obtained in human platelet-rich-plasma.

3 In contrast, PGD_2 acted as a weak and partial inhibitor in guinea-pig platelet aggregation, producing a bell-shaped dose-response relationship.

4 PGD_2 induced a dose-related antagonism of the inhibitory actions of BW245C, prostacyclin and carbacyclin on guinea-pig platelets.

5 However, PGD_2 did not antagonize the inhibitory actions of either forskolin or dibutyryl cyclic AMP on this platelet preparation.

6 The results suggest a non-specific interaction of PGD_2 with these prostanoid binding sites on guinea-pig platelets.

Introduction

Prostaglandin D_2 (PGD_2) is a potent inhibitor of platelet aggregation in human, sheep and horse plasma but is a weak inhibitor in dog, rabbit and rat plasma (Smith *et al.*, 1974; Whittle *et al.*, 1978). PGD_2 has been shown to be generated by human platelets (Oelz *et al.*, 1977) and the non-enzymatic conversion of the endoperoxide, PGH_2 to PGD_2, which can occur in plasma, is greatly enhanced by the presence of plasma protein (Hamberg & Fredholm, 1976; Christ-Hazelhof *et al.*, 1976). Pharmacological and receptor-binding studies suggest that PGE_1 interacts at the same site on human platelets as does prostacyclin and that PGD_2 acts at platelet sites distinct from those of prostacyclin and PGE_1 (Whittle *et al.*, 1978; Siegl *et al.*, 1979; Schafer *et al.*, 1979; Miller & Gorman, 1979).

The hydantoin prostaglandin analogue (BW245C) inhibits human platelet aggregation but differs considerably in structure from either prostacyclin, PGE_1 or PGD_2 (Whittle *et al.*, 1983). However, it has been suggested that BW245C interacts with similar binding sites to PGD_2 on platelets of various species (Whittle *et al.*, 1983; Town *et al.*, 1983). In the present study the potency and inhibitory characteristics of PGD_2 as an anti-aggregating agent in platelet-rich-plasma (PRP) from guinea-pig has been compared to prostacyclin, its stable analogue carbacyclin (Whittle *et al.*, 1980) and the hydantoin prostaglandin BW245C.

A preliminary account of this work has been presented to the British Pharmacological Society (Hamid & Whittle, 1984).

Methods

Male Halls guinea-pigs (350–450g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and 15 ml of blood was collected from the abdominal aorta, using needle pucture (Braunula size 1), into 5 ml plastic Sarstedt neutral tubes containing trisodium citrate (final concentration 0.315%). The blood was centrifuged (3000 r.p.m. for 2 min) at room temperature in a Petalfuge I bench centrifuge to obtain platelet-rich-plasma (PRP). The PRP was then carefully removed using a plastic syringe, mixed and

transferred to plastic universal 30 ml containers (Sterilin) and was kept 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}. All equipment used in the preparation and study of PRP and platelet-poor-plasma (PPP) was plastic or siliconized glass.

Platelet aggregation was measured in a Payton dual channel aggregation module connected to a 'W + W' recorder 1200 as described previously (Whittle *et al.*, 1978). Aliquots (0.5 ml) of PRP were incubated for 1 min at 37°C, stirred with teflon-coated magnetic stirrers at 900 r.p.m., with or without the prostaglandin under investigation, before the addition of submaximal dose of adenosine diphosphate (ADP) to just cause a non-reversible control aggregation. The concentration of ADP used for guinea-pig PRP was 2–4 μM.

The inhibition of ADP-induced aggregation was determined by preincubation (1 min at 37°C) with the prostaglandin under investigation before the addition of a sub-maximal dose of ADP. Dose-inhibition curves were constructed for PGI_2, BW245C, carbacyclin and PGD_2 and the ID_{50} (dose causing 50% inhibition) was calculated as the dose required to reduce the aggregation to 50% of its control amplitude.

To study the interaction of PGD_2 with the other prostaglandins, doses producing approximately 80–95% inhibition of ADP-induced aggregation were chosen (PGI_2, 4 ng ml^{-1}; BW245C, 6 ng ml^{-1}; carbacyclin, 40 ng ml^{-1}). PRP was pre-incubated (1 min at 37°C) with PGD_2 (100–2000 ng ml^{-1}) before adding the appropriate concentration of the prostaglandin under investigation for a further 1 min incubation at 37°C. The sub-maximal dose of ADP was then added. In further studies, full dose-inhibition curves were constructed for BW245C in the presence of PGD_2 (500, 2000 and 5000 ng ml^{-1}). The interaction of PGD_2 with forskolin (10 μg ml^{-1}) and dibutyryl adenosine 3'5'-cyclic monophosphoric acid (dibutyryl cyclic AMP; 2.9 mg ml^{-1}) was also studied.

To prepare human PRP, approximately 200 ml of human blood was freshly collected following venepuncture in volunteers (who had not taken any non-steroidal anti-inflammatory agents for 1 week before the study) into a plastic 150 cm^2 tissue culture flask (Corning) containing trisodium citrate (3.15% w/v solution to give a final citrate concentration of 0.315% w/v). Whole blood was then centrifuged in plastic tubes at 1100 r.p.m. (200 *g*) in an MSE bench centrifuge for 15–20 min at room temperature. The PRP was then carefully removed using a plastic syringe, mixed and transferred to plastic universal 30 ml containers (Sterilin) and kept at room temperature. Platelet aggregation in human PRP was carried out as described for guinea-pig PRP above.

Drugs

Prostacyclin as the sodium salt was freshly dissolved in 1 M Tris Buffer (pH 9.6 at 4°C) and stored on ice; subsequent dilutions were made with 50 mM Tris buffer (pH 9 at 4°C) immediately before use. Prostaglandin D_2 and carbacyclin, (supplied by the Upjohn Company, Kalamazoo) and the hydantoin prostaglandin, 5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl)-hydantoin (BW245C; from the Department of 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. Forskolin (Calbiochem-Behring Corp., La Jolla, Calif.) was stored in ethanol (10 mg ml^{-1}) and diluted when required with 50 mM Tris buffer. Adenosine diphosphate (Sigma Chemical Co.) and dibutyryl adenosine 3'5'-cyclic monophosphoric acid (Sigma Chemical Co.) were dissolved in distilled water when required and kept on ice.

Statistical analysis

Results are expressed as mean ± standard error of 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. $P < 0.05$ was taken as significant.

Results

Pre-incubation of prostacyclin (PGI_2), BW245C and carbacyclin with guinea-pig or human PRP caused a dose-dependent inhibition of platelet aggregation induced by ADP (Figure 1). The ID_{50} for prostacyclin, BW245C and carbacyclin in guinea-pig PRP was

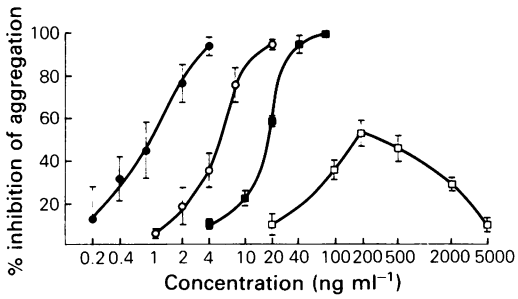

Figure 1 Inhibition of ADP-induced platelet aggregation in guinea-pig platelet-rich-plasma by prostacyclin (●), BW245C (○), carbacyclin (■) and prostaglandin D_2 (□) following 1 min pre-incubation at 37°C. Results show as means, with vertical lines representing s.e.means, from 3–6 experiments.

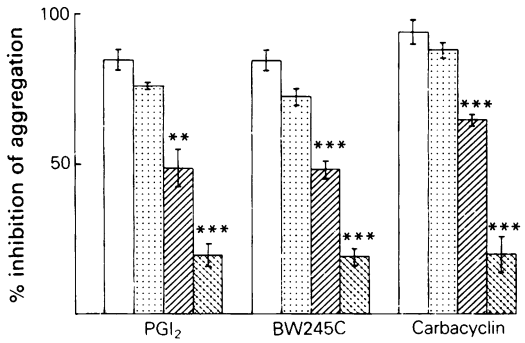

Figure 2 Antagonism of prostanoid-induced inhibition of platelet aggregation in guinea-pig PRP by prostaglandin D_2 (PGD_2). Inhibition of platelet aggregation by prostacyclin (PGI_2, 4 ng ml^{-1}; $n = 6$), BW245C (6 ng ml^{-1}; $n = 6$) and carbacyclin (40 ng ml^{-1}; $n = 6$) was antagonized by pre-incubation (1 min) with PGD_2 (100–2000 ng ml^{-1}) in a dose-related manner. Control, open columns; plus PGD_2: 100 ng ml^{-1} stippled columns; 500 ng ml^{-1}, hatched columns, 2000 ng ml^{-1}, cross-hatched columns. Results, expressed as % inhibition of platelet aggregation compared to the initial controls, are shown as the mean ± s.e.mean (vertical lines) of n experiments. ** $P < 0.01$; *** < 0.001.

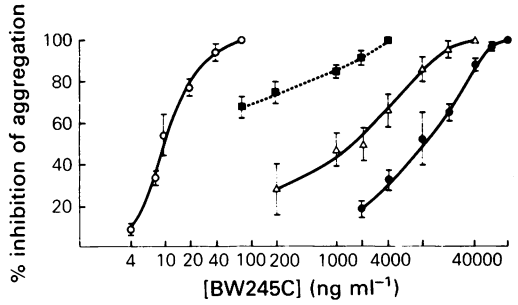

Figure 3 Antagonism of BW245C (4–80 ng ml^{-1}) induced inhibition of platelet aggregation in guinea-pig PRP by prostaglandin D_2 (PGD_2; 500–5000 ng ml^{-1}). The control BW245C (○, $n = 6$) dose-inhibition curve was shifted to the right in the presence of PGD_2 in a dose-related manner; PGD_2 (500 ng ml^{-1} (■, $n = 3$), 2000 ng ml^{-1} (△, $n = 4$) and 5000 ng ml^{-1} (●, □ = 3). Results, expressed as % inhibition of platelet aggregation compared to initial controls, are shown as the mean of n experiments with vertical lines representing s.e.means.

approximately similar to those in human PRP (Table 1). Thus, guinea-pig PRP provides one of the more sensitive animal platelets to the anti-aggregating actions of these prostanoids (see Whittle *et al.*, 1978) and is comparable to human platelets in this respect.

In contrast, PGD_2 gave a bell-shaped dose-response relationship, with a maximum inhibition of aggregation of 53.2 ± 6% ($n = 7$) at 200 ng ml^{-1} which was significantly lower at 2000 ng ml^{-1} (29.2 ± 3.4% inhibition, $n = 8$; $P < 0.01$) and 5000 ng ml^{-1} (10.4 ± 3.5% inhibition, $n = 7$; $P < 0.01$), as shown in Figure 1. Furthermore, pre-incubation (1 min) with PGD_2 (200–5000 ng ml^{-1}) caused a significant ($P < 0.01$) dose-related antagonism of the near-maximal anti-aggregating actions of PGI_2 (4 ng ml^{-1}), carbacyclin (40 ng ml^{-1}) and BW245C (6 ng ml^{-1}), as shown in Figure 2. The degree of antagonism with PGD_2 (500–2000 ng ml^{-1}) of the actions of PGI_2, carbacyclin and BW245C was comparable (Figure 2).

In further studies, the dose-inhibition curve of BW245C and of carbacyclin was shifted to the right in the presence of PGD_2 (500–5000 ng ml^{-1}) in a dose-related manner. Thus the ID_{50} of BW245C was significantly increased 350 fold from 7.3 ± 3 ng ml^{-1} to 2.5 ± 0.6 μg ml^{-1} ($P < 0.001$), by PGD_2 (2000 ng ml^{-1}) as shown in Figure 3, and that of carbacyclin 1000 fold from 18.6 ± 0.6 ng ml^{-1} to 20.3 ± 9.8 μg ml^{-1} ($P < 0.05$) in the presence of PGD_2 (2000 ng ml^{-1}) (results not illustrated). Since PGD_2 has intrinsic activity as an inhibitor of platelet aggregation, especially at the lower concentrations, the nature of the dose-inhibition relationships was complex. Thus, in the presence of PGD_2, the minimal effective concentrations of the anti-aggregating agents BW245C or carbacyclin, and the shape of their concentration-response curves were dependent on the concurrent concentration of PGD_2 (Figure 3).

Pre-incubation (1 min) with PGD_2 (2000 and

Table 1 Inhibition of ADP-induced platelet aggregation by prostacyclin (PGI_2), BW245C, carbacyclin and prostaglandin D_2 (PGD_2).

	ID_{50} (ng ml^{-1})			
	PGI_2	*BW245C*	*Carbacyclin*	*PGD_2*
Human	0.5 ± 0.1(15)	1.4 ± 0.1(10)	11.0 ± 3.0(10)	11.0 ± 1.6(15)
Guinea-pig	0.8 ± 0.2 (6)	7.3 ± 3.0(14)	18.6 ± 0.6 (5)	*162.0 ± 30.7 (5)

Results, expressed as concentration of prostanoid causing 50% inhibition of ADP-induced platelet aggregation in human and guinea-pig platelet-rich-plasma (PRP), are mean ± s.e.mean from (number in parentheses) experiments.
*PGD_2 produced a bell-shaped curve in guinea-pig PRP.

5000 ng ml^{-1}) did not cause any antagonism of the near-maximal anti-aggregating actions of forskolin (10 μg ml^{-1}) and dibutyryl cyclic AMP (2.9 mg ml^{-1}), as shown in Figure 4. Indeed, PGD_2 in these concentrations significantly ($P<0.01$) enhanced the anti-aggregating actions of both forskolin and dibutyryl cyclic AMP.

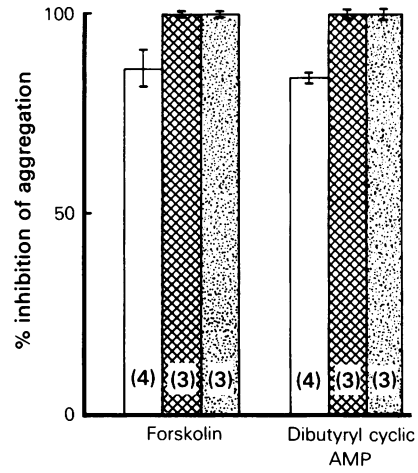

Figure 4 Failure of prostaglandin D_2 (PGD_2; 2–5 μg ml^{-1}) to antagonize forskolin (10 μg ml^{-1}) and dibutyryl adenosine 3'5'-cyclic monophosphoric acid (dibutyryl cyclic AMP; 2.9 mg ml^{-1}) induced inhibition of platelet aggregation in guinea-pig PRP. Controls, open columns; plus PGD_2: 2 μg ml^{-1}, cross-hatched columns; 5 μg ml^{-1}, stippled columns. Results, expressed as % inhibition of platelet aggregation compared to the initial controls, are shown as the mean of *n* (number in parentheses) experiments with vertical lines representing s.e.mean.

Discussion

The response and potency of various prostaglandins in guinea-pig platelets aggregated with ADP has not previously been described in detail. In this study, a technique for the collection of blood from the guinea-pig abdominal aorta has been utilized for the preparation of platelet-rich-plasma (PRP). The inhibition of ADP-induced aggregation of guinea-pig platelets with prostacyclin, BW245C and carbacyclin had comparable potency to that with human PRP (Table 1). Thus, guinea-pig PRP provides one of the more sensitive animal platelets to the anti-aggregating actions of these prostanoids. In contrast, PGD_2 provided a different profile in the guinea-pig platelets, acting as a weak partial inhibitor and producing a bell-shaped dose-response relationship, whereas in human PRP, PGD_2 acts as a full potent inhibitor of ADP-induced aggregation.

Previous studies have suggested that BW245C can interact with PGD_2 binding sites on platelets from different species (Whittle *et al.*, 1983; Town *et al.*, 1983). However, in guinea-pig PRP, where PGD_2 was found to be only a weak partial inhibitor of platelet aggregation, BW245C acted as a potent full inhibitor of platelet aggregation. Thus, in the guinea-pig platelets BW245C does not simply act as a PGD_2 mimetic, but possibly acts at sites distinct from those of PGD_2, perhaps at the prostacyclin-sensitive site.

Because of the characteristics of the PGD_2 dose-response curves, further studies with PGD_2 as a potential antagonist of the inhibitory actions of the other prostanoids were conducted. The interpretation of the interactions of PGD_2 is complicated since it does not behave as a typical partial agonist, exhibiting a bell-shaped dose-response curve rather than reaching a plateau inhibitory response at submaximal doses of inhibition. PGD_2 antagonized the inhibitory actions of not only BW245C, but also of prostacyclin and carbacyclin (Figure 2). Furthermore, the rightward shift of the control dose-response curves in the presence of PGD_2 was far greater than one would expect of a competitive antagonist (Figure 3). Thus, PGD_2 appears to act as a non-specific antagonist of the inhibitory actions of various prostanoids in guinea-pig platelets.

The diterpene, forskolin, has been shown to be a potent general activator of adenylate cyclase in membrane preparations and in intact cells from a variety of tissues, and thus an elevator of cyclic AMP levels (Seamon *et al.*, 1981). Thus, forskolin is a useful agent for the general activation of adenylate cyclase and hence for the investigation of the relationship of cyclic AMP levels to physiological functions in a variety of systems. Platelet aggregation can be inhibited by any agent or process which elevates platelet intracellular cyclic AMP levels (Salzman & Levin, 1971; Haslam, 1975). Elevated cyclic AMP levels appear to regulate platelet cytoplasmic calcium levels by stimulating calcium removal by a membrane system, probably the dense tubular system (Kaser-Glanzmann *et al.*, 1977). Thus forskolin has been shown to be a potent inhibitor of aggregation of human platelets induced by a wide variety of aggregating agents (Siegel *et al.*, 1982). In this study, the platelet inhibitory actions of forskolin, in contrast to those of PGI_2 and carbacyclin, were not antagonized by PGD_2. Since forskolin circumvents receptor interaction and stimulates adenylate cyclase more directly, these findings suggest that PGD_2 does not interact by non-specifically suppressing adenylate cyclase activation. Further, PGD_2 did not antagonize the anti-aggregating actions of dibutyryl cyclic AMP, the stable and more lipophilic analogue of cyclic AMP, which is not metabolized by phosphodiesterases. Thus

PGD_2 does not appear to act as a direct antagonist of the actions of intracellular cyclic AMP in these platelets.

The underlying mechanism of the bell-shaped curve and the antagonist actions of PGD_2 in guinea-pig platelets is not clear. One possibility is that there are two types of PGD_2 receptors, one specific for inhibition and the other, at high concentrations, for stimulation of platelet aggregation. Another possibility is that auto-inhibition of the PGD_2 receptor occurs; as the concentration of PGD_2 increases, it antagonizes its own responses.

PGD_2 may also cause a non-specific desensitization and prostanoid receptor down regulation. Blair *et al.* (1982) have shown carbacyclin to cause a decrease in affinity of the prostacyclin-receptor interaction, as well as a decrease in the total receptor numbers of cultures of NCB-20 cells. Exposure to carbacyclin (1μM) over a 4–16 h period also caused a progressive decrease in the prostaglandin-dependent activaton of adenylate cyclase in cell homogenates. However, such desensitization with PGD_2 seems unlikely in the present study since PGD_2 was in contact with the platelets for only 1 min before the addition of the anti-aggregating prostanoids.

At high concentrations PGD_2 may also interact with other stimulatory and pro-aggregatory receptors on guinea-pig platelets, such as those for thromboxane A_2 (Svensson *et al.*, 1975; Armstrong *et al.*, 1983), although PGD_2 did not itself induce aggregation in this species and is a full inhibitor of platelet aggregation in most other species (Whittle *et al.*, 1978; 1983). The complex interactions between the potential pro- and anti-aggregating properties with PGD_2 may thus be unmasked by the use of selective prostanoid-receptor antagonists.

This study shows PGD_2 to be an antagonist of the anti-aggregating actions of several prostanoids but not a non-specific inhibitor of adenylate cyclase or antagonist of cyclic AMP in guinea-pig platelets. It is not yet known whether such antagonistic effects of PGD_2 reflect direct interactions of the binding or receptor sites for the other anti-aggregating prostanoids on the platelets. The development of specific prostanoid receptor antagonists would assist in the interpretation of the current findings.

References

ARMSTRONG, R.A., JONES, R.L. & WILSON, N.H. (1983). Ligand binding to thromboxane receptors on human platelets: correlation with biological activity. *Br. J. Pharmac.*, **79**, 953–964.

BLAIR, I.A., LEIGH, P.J. & MACDERMOT, J. (1982). Desensitization of prostacyclin receptors in a neuronal hybrid cell line. *Br. J. Pharmac.*, **77**, 121–127.

CHRIST-HAZELHOF, E., NUGTEREN, D.H. & VAN DORP, D.A. (1976). Conversion of prostaglandin endoperoxides by glutathione-s-transferases and seum albumins. *Biochim. biophys. Acta*, **450**, 450–461.

HAMBERG, M. & FREDHOLM, B.B. (1976). Isomerization of prostaglandin H_2 into prostaglandin D_2 in the presence of serum albumin. *Biochem. biophys. Acta*, **431**, 189–193.

HAMID, S. & WHITTLE, B.J.R. (1984). Prostaglandin D_2 acts as a 'partial antagonist' in guinea-pig platelets. *Br. J. Pharmac.*, **81**, 96P.

HASLAM, R.J. (1975). Roles of cyclic nucleotides in platelet function. In *Ciba Geigy Foundation Symposium*, vol. 35, pp. 121–151. Amsterdam: Elsevier.

KASER-GLANZMANN, R., JAKABOVA, M., GEORGE, J.N. & LUSCHER, E.F. (1977). Stimulation of calcium uptake in platelet membrane vesicles by adenosine 3', 5'-cyclic monophosphate and protein kinase. *Biochim. biophys. Acta*, **466**, 429–440.

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.

OELZ, O., OELZ, R. & KNAPP, H.P. (1977). Biosynthesis of prostaglandin D_2. I Formation of prostaglandin D_2 by human platelets. *Prostaglandins*, **13**, 225–234.

SALZMAN, E.W. & LEVIN, L. (1971). Cyclic 3', 5'-Adenosine monophosphate in human blood platelets. *J. clin. Invest.*, **50**, 131–141.

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.

SEAMON, K.B., PADGETT, W. & DALY, J.W. (1981). Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. natn. Acad. Sci. U.S.A.*, **78**, 3363–3367.

SIEGL, A.M., DALY, J.W. & SMITH, J.B. (1982). Inhibition of aggregation and stimulation of cyclic AMP generation in intact human platelets by the diterpene forskolin. *Mol. Pharmac.*, **21**, 680–687.

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.

SMITH, J.B., SILVER, M.J., INGERMAN, C.M. & KOCSIS, J.J. (1974). Prostaglandin D_2 inhibits the aggregation of human platelets. *Thrombosis Res.*, **5**, 291–299.

SVENSSON, J., HAMBERG, M. & SAMUELSON, B. (1975). Prostaglandin endoperoxides IX Characterisation of rabbit aorta contracting substance (RCS) from guinea-pig lung and human platelets. *Acta physiol. scand.*, **94**, 222–228.

TOWN, M-H., CASALS-STENZEL, J. & SCHIKINGER, E. (1973). Pharmacological and cardiovascular properties of a hydantoin derivative, BW245C, with high affinity and selectivity for PGD_2 receptors. *Prostaglandins*, **25**, 13–28.

WHITTLE, B.J.R., MONCADA, S., MULLANE, K. & VANE, J.R. (1983). Platelet and cardiovascular activity of the hydan-

toin 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.

WHITTLE, B.J.R., MONCADA, S., WHITING, F. & VANE, J.R. (1980). Carbacyclin – a potent stable prostacyclin analogue for the inhibition of platelet aggregation. *Prostaglandins*, **19**, 605–627.

(Received November 26, 1984.
Accepted December 19, 1984.)