Classification of platelet and vascular prostaglandin D₂ (DP) receptors: estimation of affinities and relative efficacies for a series of novel bicyclic ligands

With an Appendix on Goodness-of-fit analyses

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1 The DP receptors located on platelets and vasculature were examined in a human washed platelet preparation and in isolated rings of rabbit external jugular vein.

2 A series of eight novel bicyclic compounds were studied for their effects in the two assays. Seven produced agonism, inhibition of aggregation or vascular relaxation, and one compound was 'silent' in both assays.

3 The operational model of agonism (Black & Leff, 1983) was fitted simultaneously to concentration-effect curve data for the seven agonist compounds. The affinity and efficacy estimates so obtained were tested for similarity between the two tissues by analysis of variance, showing that the model could be fitted to both sets of data by assuming the same relative affinity and efficacy values. However, absolute affinity estimates were consistently lower in the vascular preparation.

4 Analysis of two of the seven agonists as antagonists was also possible. This provided pK_B estimates which supported the agonist affinity estimates. The eighth compound was also analysed as an antagonist. It, like the other seven, demonstrated a difference in affinity between the two tissues.

5 The results of this study support the view that platelet and vascular DP receptors are similar, assuming that the systematic difference in affinity estimates for the series of compounds between the two tissues is the consequence of receptor micro-environment and/or accessory binding site differences.

Keywords: Prostaglandin D₂; DP-receptors; classification; dissociation constant; efficacy; 572C85; 192C86; BW A868C

Introduction

In a previous paper (Giles *et al.*, 1989) we described a study of platelet and vascular prostaglandin-D₂ (DP) receptors using a newly available antagonist, BW A868C. The results of that study indicated that the DP-receptors in those two locations belonged to the same class. However, this conclusion had to remain provisional because demonstration of simple competitive antagonism by BW A868C was precluded in the vascular assay due to multiple actions on the part of the available agonists. Thus, while a reliable estimate of the dissociation constant for BW A868C could be obtained for the platelet receptor, this was not possible for the vascular smooth muscle receptor. Furthermore, as shown in the present study, when DP-receptor agonists of appropriate specificity were identified, which should have allowed a quantitative analysis of antagonism by BW A868C to be performed in the vascular assay, further problems of a kinetic nature were revealed.

In an attempt to solve some of these problems we have now identified and analysed the actions of a series of novel bicyclic agonist and antagonist analogues. The objective of this study has been to obtain quantitative information, affinity and efficacy estimates, for these compounds in platelet and vascular assays and thereby provide a more secure basis for the classification of DP-receptors in these tissues.

Methods

Tissue preparations

Human washed platelets Blood was drawn from healthy male and female volunteers who had not taken any drugs known to affect platelet function for at least two weeks and collected into sodium citrate (3.15% w/v) in proportion 9:1 w/v. Prostacyclin-washed platelets suspended in Tyrode buffer were prepared as previously described (Giles *et al.*, 1989). Indomethacin (5 µg ml⁻¹) and fibrinogen (400 µg ml⁻¹) were added to the platelet suspension at the start of each experiment. Aggregation responses were measured in 0.5 ml aliquots of the suspension maintained at 37°C and stirred with a metal bar at 900 r.p.m. in 300 BD-S Payton dual channel aggregometers and recorded on Gould BS 272 pen recorders.

Rabbit jugular vein External jugular veins were removed from male New Zealand White rabbits (2.4–3.0 kg) killed by injection of pentobarbitone sodium (Sagatal, 60 mg kg⁻¹) into a marginal ear vein. The vessels were cannulated, which caused the removal of the endothelium (Leff *et al.*, 1987), and then cleared of adhering tissue. Three 5 mm wide ring segments were obtained from each vein and each was suspended between two wire hooks, one attached to a Grass FT03C force displacement transducer and the other to a stationary support in a 20 ml organ bath containing Krebs solution (pH 7.4) of the following composition (mM): NaCl 118.4, NaHCO₃ 25.0, KCl 4.8, KH₂PO₄ 1.2, MgSO₄ 1.2, glucose 11.1, CaCl₂ 2.5, maintained at 37°C and continuously gassed with 95% O₂:5% CO₂. Responses were measured as

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changes in isometric force and recorded on Gould BW 272 pen recorders.

Experimental protocols

Human washed platelets Prostaglandin receptor agonists were incubated in the platelet suspension for 6 min before addition of ADP (50 μ M). Previous experiments showed that, for all agonists used in this study, this was sufficient time to reach equilibrium. The reproducibility of the ADP-induced aggregation response was checked at intervals throughout the experiment. When an antagonist was used it was added 2 min before the agonist. Responses to the agonist were expressed as percentage inhibition of the control ADP-induced aggregation. The total volume of solutions added to the suspension was always less than 5% of the platelet suspension volume.

Rabbit jugular vein Indomethacin (1 μ g ml⁻¹) and the TP receptor antagonist, BM 13.177 (30 μ M) were used routinely in all experiments, as described previously (Giles *et al.*, 1989). Initially, jugular vein ring segments were subjected to a force of 0.75 g then allowed to stabilize for a period of 60 min during which time preparations were washed three times. BM 13.177 was incubated for 60 min then histamine (1 μ M) was added in order to produce tone. After establishing a stable contraction, relaxation concentration-effect curves were obtained with prostaglandin receptor agonists. A separate experiment demonstrated that the histamine contraction was generally stable (<5% loss of tone) for the period of time required to construct a concentration-effect curve. Tissues in which there was an initial rapid loss of tone were discarded. Responses were expressed as percentage relaxations of the histamine-induced force. Antagonist studies followed a paired curve design. This was necessary because of substantial inter-tissue variation in the position of prostaglandin receptor agonist concentration-effect curves. Preparations were washed thoroughly following the first concentration-effect curve and allowed to re-stabilize. After re-introduction of BM 13.177, antagonists were incubated for 60 min before constriction with histamine (1 μ M) followed by a second prostaglandin agonist concentration-effect curve. A separate experiment established that first and second histamine concentration effect curves, and first and second control prostaglandin agonist concentration-effect curves were superimposable (data not shown).

Analysis of data

Curve fitting A logistic function of the form:

$$E = \frac{\alpha[A]^m}{[A]_{50} + [A]^m} \quad (i)$$

in which α , $[A]_{50}$ and m are the asymptote, location and slope parameters respectively, was fitted to each concentration-effect curve in order to generate average parameter estimates for each group of curves.

Estimation of antagonist affinity: (i) *Single curve design* A one-way analysis of variance tested for treatment effects on the computed estimates of α and m . In the platelet study, inhibition of aggregation greater than 100% of the ADP aggregation cannot be obtained, therefore analysis of variance on the asymptote (α) was inappropriate. Tests for parallelism relied on slope analysis in this case. If the antagonist did not significantly modify these parameters then computed $\log_{10} [A]_{50}$ values were fitted to the following form of the Schild equation (Black *et al.*, 1985):

$$\log_{10}[A]_{50} = \log_{10}[A]_{50}^c + \log_{10}(1 + [B]^n/K_B) \quad (ii)$$

where $[A]_{50}^c$ is a control A_{50} value, $[B]$ is the concentration of antagonist, K_B is its equilibrium dissociation constant and n is its order of reaction with the receptor. If n was not

significantly different from unity, indicating simple competition, it was constrained to this value in order to obtain an estimate of K_B . The dissociation constant was actually estimated as pK_B ($-\log_{10} K_B$). In this experimental design a complete study of antagonism was conducted on each suspension or tissue and so equation (ii) was used to provide an estimate of pK_B on each occasion. The average of these values is quoted in the text together with the standard error.

(ii) *Paired curve design* Paired t tests were performed on the differences between computed α and m estimates for each pair of concentration-effect curves. If the antagonist did not significantly modify α or m then the Schild equation was fitted to the computed $r - 1$ values for each pair of curves:

$$\log(r - 1) = n \log [B] - \log K_B \quad (iii)$$

where r is the dose-ratio (A_{50}^c/A_{50})

Ideally, a complete study of antagonism should have been conducted on each day (Giles *et al.*, 1989), thus allowing a set of independent estimates of pK_B and n to be obtained. However in the present case, on some occasions data from the jugular vein had to be rejected due to a fading histamine contraction, or because the efficacy of the agonist in that tissue was too small to be defined (presumably due to low receptor reserve). As a result, a complete set of concentration-ratio data was often unobtainable, meaning that a daily analysis was precluded. Therefore, concentration-ratio data obtained over successive days were pooled and fitted simultaneously using the Schild equation. If n was not significantly different from unity, indicating simple competition, it was constrained to this value to obtain an estimate of pK_B together with the standard error. The error estimates on n and pK_B so obtained include fitting error as well as experimental error and are larger than would have been the case had daily estimates been possible. However, the resulting lack of stringency does not compromise interpretation in the present study, as will be seen.

Estimation of agonist affinity and efficacy

The operational model of agonism (Black & Leff, 1983) was fitted to concentration-effect curve data sets where:

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (iv)$$

in which E_m is the maximum effect of a full agonist, τ is the efficacy, K_A is the dissociation constant for the agonist, and n is the slope or sensitivity index for the occupancy-effect relation. Eq. (iv) was fitted to each data set assuming a common K_A but a separate τ value for each curve. The rationale for this, and the associated estimation of error has been described elsewhere (Leff *et al.*, 1990).

As all of the agonists in question behaved as partial agonists a full agonist was required for comparison. In terms of the operational model a standard full agonist is used to provide estimates of E_m and n . This is because for an agonist with high efficacy the shape of the concentration-effect relation is identical to the shape of the occupancy-effect relation (Leff, 1987) which in turn is characterized by the parameters E_m and n . Algebraically, the operational model equation describing a full agonist is a simplified version of equation (iv):

$$E = \frac{E_m [A]^n}{(K_A/\tau)^n + [A]^n} \quad (v)$$

which is of a logistic form (Leff, 1987). Therefore, E_m and n can be estimated by fitting the full agonist data directly to a logistic function.

Practically, the high efficacy of the standard agonist, that is, its 'fullness' should be checked with an irreversible antagonist. The lack of such an agent for the present receptors meant that evidence for high efficacy had to be sought

elsewhere. Such evidence is available when an agonist naturally varies in its potency ($[A_{50}]$) in a particular tissue without its maximal effect varying. Assuming that such variation is due to receptor reserve differences between tissues (e.g. to a difference in receptor density or the efficiency of transduction), the implication is that, despite low reserve in some tissues, the agonist can still produce a maximum effect and, therefore, on average it has high intrinsic efficacy. As described later, the standard full agonist used here (BW245C) demonstrated these characteristics.

In order to assess goodness-of-fit and the similarity or otherwise of affinity (pK_A) and efficacy (τ) estimates between tissues, initially a curve was fitted to each individual concentration-effect curve; this allowed estimation of the sum-of-squares associated with fitting. Thereafter, for each tissue, a single curve was fitted to all the data for each agonist under the imposition of various constraints on the parameter estimates (see Appendix).

Drugs

PGE₂ (Cayman Chemical, Denver, Colorado, U.S.A.), 9,11-dideoxy, 9 α -11-methanoepoxy-PGE₂ (U46619) (Cayman), Epoprostenol sodium Grade 1 (PGI₂) (Wellcome Research Laboratories, Beckenham, Kent), fluprostenol sodium (gift from Coopers Animal Health Ltd., Berkhamstead, Herts), BW 245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl)-hydantoin), BW A868C(($\pm$)-3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin), BM13.177 (4-[2-(benzenesulphonamido)-ethyl]-phenoxyacetic acid) and Compound I-VIII (Figure 1) (Wellcome), indomethacin (Sigma Chemical Co. St. Louis, MO, U.S.A.), adenosine 5'-diphosphate (ADP) (Sigma), histamine HCl (Sigma), fibrinogen (KabiVitrum Stockholm, Sweden), phenoxybenzamine hydrochloride (Smith, Kline and French, Welwyn Garden City, Herts), atropine sulphate (Sigma).

PGI₂ solutions were made daily in 1 M Tris buffer pH 8.4. All other prostaglandins and their analogues were dissolved initially in ethanol and stored as 20 mM solution at -22°C. Further dilutions were made daily in distilled water, except for BW A868C which required 0.5 M Tris buffer pH 8.4 for the 2 mM dilution. All dilutions were stored on ice. Indomethacin 10 mg ml⁻¹ was dissolved in 0.25 M Tris buffer pH 8.4; histamine HCl 1 mM was prepared in aqueous solution neutralised to pH 7 with 10 mM NaOH; phenoxybenzamine was dissolved in absolute ethanol; ADP, fibrinogen and atropine were aqueous solutions.

At final concentrations to which the tissues were exposed, none of the vehicles influenced tissue responsiveness.

Results

DP receptor effects of compounds I-VIII

Figure 2 illustrates the inhibition of aggregation (a) and vasorelaxation (b) elicited by compounds I-VIII (see Figure 1). The compounds appeared to display a range of intrinsic efficacies and the receptor reserve was apparently higher in the platelets than in the jugular vein (assuming that the receptors are similar in both tissues). Each of the compounds which displayed agonism (I-VII) were antagonized by BW A868C, the previously classified DP receptor antagonist (Giles *et al.*, 1989) in a concentration (100 nM) 100 $\times$ its receptor dissociation constant. In the platelet assay, BW A868C elicited an approximately 100 fold parallel rightward displacement of each agonist concentration-effect curve. In the jugular vein assay, this concentration of BW A868C also elicited a rightward displacement of the agonist curves but produced slowing of agonist-induced responses to the extent that steady-state effects could not be measured.

A complete study of antagonism by BW A868C was conducted in the platelet assay using compound I - 572C85

Figure 1 The structures of the eight novel bicyclic prostaglandin analogues used in this study, together with the symbols used in Figures 2 and 5.

(such a study was not possible in the jugular vein assay). In the platelet, the antagonist (3–300 nM) produced parallel rightward displacements of compound I concentration-effect curves (Figure 3a, as assessed by one-way analyses of variance, see Methods). Figure 3b shows the associated Schild plot of all the concentration-ratios obtained. As explained in the Methods section a separate analysis of antagonism was conducted on each day, providing an estimate of the Schild plot slope and a pA_2/pK_B on each occasion. The average slope value over five days was 1.39 ± 0.17 which is significantly higher than unity ($0.025 < P < 0.05$). The average pA_2 value was 8.73 ± 0.13 (a pK_B value is not appropriately quoted under these conditions, see Jenkinson, 1991).

Receptor selectivity of compounds I-VIII

Compounds I-VIII were also studied (on at least one occasion) for possible effects at other prostanoid receptors. All experiments were performed in the presence of 10 μ M BW A868C to prevent binding to the DP receptor. None of the compounds, at concentrations up to 10 μ M, exerted any effect on baseline tone in the rabbit jugular vein preparation nor on histamine (1 μ M)-induced tone in that assay. It has previously been shown that this jugular vein preparation contains TP-receptors which mediate vasoconstriction, IP-receptors and EP₂-receptors which mediate vasorelaxation (Giles *et al.*, 1989; Lawrence *et al.*, 1989). Also, in guinea-pig ileum and rat fundus preparations, which contain respectively EP₁-receptors (Coleman *et al.*, 1984) and FP-receptors (Dong *et al.*, 1986) mediating smooth muscle contraction, compounds I-VIII were without effect at concentrations up to 10 μ M.

Antagonist actions were also examined, again at least on one occasion for each compound, in each of these three assays under the same conditions. U46619, prostacyclin and BW 245C concentration-effect curves in the jugular vein assay were unaffected by the compounds, as were concentration-effect curves to prostaglandin E₂ in guinea-pig ileum and to the selective FP-receptor agonist, fluprostenol (Dukes *et al.*, 1974; Coleman *et al.*, 1984) in rat fundus. For one of

Figure 2 The inhibitory effects of compounds I-VIII on (a) human washed platelet aggregation and (b) histamine-contracted rabbit jugular vein. Responses are the mean of 3–6 replicate curves. See Figure 1 for key to symbols. Vertical bars show s.e.mean. For clarity, error bars have been included only at the highest agonist concentrations.

the compounds, compound VI(192C86), actions at other, non-prostanoid receptors (α_1 , α_2 , β_1 , β_2 adrenoreceptors, H_1 , H_2 histamine receptors, D_1 and D_2 dopamine receptors, muscarinic and purine receptors) were also excluded in appropriate assays.

Quantitative analysis of agonism and antagonism

Agonism Assuming that the data illustrated in Figure 2 are associated with actions on a single homogeneous population of DP-receptors, the following analyses of agonism and antagonism were performed.

As explained in Methods, it was necessary first to analyse a 'full' agonist in order to obtain estimates of E_m and n . BW 245C, a DP receptor agonist (Town *et al.*, 1983; Whittle *et al.*, 1983), was selected because it demonstrated complete inhibition of platelet aggregation and complete relaxation in the jugular vein assay with variations of up to 1 logarithmic unit in the location ($[A_{50}]$) of its concentration-effect curve in different preparations of the same tissue. This indicated that BW 245C possesses a high intrinsic efficacy at DP-receptors. The logistic function (equation i) was fitted to 13 replicated BW 245C concentration-effect curves obtained in the platelet assay, and to 16 replicate curves in the jugular vein assay. In the jugular vein all the BW 245C concentration-effect curves were shifted rightwards by BW A868C, demonstrating that agonism was being mediated through the DP receptor, and

Figure 3 (a) Antagonist effects of BW A868C on 572C85 (compound I) concentration-effect curves in human washed platelets. BW A868C was used at the following concentrations: zero (○), 3 nM (●), 10 nM (□), 30 nM (■), 100 nM (△). Responses are the mean of 3–5 replicate curves. (b) Schild plot antagonism of 572C85 by BW A868C in human platelets. The line through the data was calculated as described in the methods section.

not through actions at the EP_2 receptor, since BW 245C has efficacy at both DP and EP_2 receptors (Giles *et al.*, 1989). Average parameter estimates were as follows: platelet: $E_m = 99.85 \pm 0.84$, $n = 1.17 \pm 0.07$; jugular vein: $E_m = 100.70 \pm 1.13$; $n = 1.06 \pm 0.03$. In subsequent model-fits E_m and n were constrained to these values. Average data, along with the average logistic curve fits, are shown in Figure 4.

Concentration-effect curve data for agonists I-VII from both assays were then fitted simultaneously using the operational model of agonism (equation iv). This provided estimates of affinity (pK_A) and efficacy (τ) for each agonist in each tissue (Table 1). These estimates were then tested for their similarity between tissues by imposing a number of selected constraints on the model parameters and analysing the effect of these constraints on the residual sums-of-squares associated with model-fitting.

The results of these analyses are described in full in the Appendix. In brief, it was found that: (i) a significant worsening of fit was obtained when the affinity (pK_A) of each agonist was assumed to be the same in the two tissues, although no worsening occurred when the pK_A values for each agonist were assumed to differ by a constant increment between the two tissues; (ii) a significant worsening of fit occurred when the efficacy (τ) value for each agonist was assumed to be the same in each tissue, but no worsening occurred when a constant ratio was assumed between the values for each agonist acting in the two tissues. Therefore,

the series of agonists were concluded to express the same relative affinities and efficacies in the two tissues. Figure 5 illustrates the average experimental data and model-fitted lines obtained assuming that the relative pK_A and τ values were the same for each agonist (i.e. Fit 9 on Table A1, Appendix).

Figure 4 Concentration-effect curves to BW 245C showing (a) inhibition of ADP-induced human platelet aggregation (13 replicates) and (b) inhibition of histamine contraction in rabbit jugular vein (16 replicates). The lines through the data are the average logistic fit (see Methods, curve fitting), which resulted in the following parameter estimates: platelet: $\log[A_{50}] = -8.17$, α 100, m 1.17; jugular: $\log[A_{50}] = -7.24$, α 100, m 1.06. Vertical bars show s.e.mean.

Antagonism On certain occasions when, presumably, receptor reserve was low in the jugular vein, compounds VI and VII produced no agonist effects and, under these circumstances, they could be analysed as competitive antagonists. On these occasions the maximum effect of a saturating concentration of 572C86 (a partial agonist in this tissue) was

Figure 5 Model-fitted lines using data from Figure 2 which shows the inhibitory effects of compounds I-VII on (a) human platelet aggregation and (b) contraction of rabbit jugular vein. The lines are the result of fitting the operational model of agonism (Eq. iv) to the data (Fit 9 on Table A1, Appendix). A between-tissues fixed increment in both affinity (K_A 0.47 $\log_{10}$ units) and efficacy (τ , 4.04 fold) was allowed, since initial estimates of both parameters were systematically lower in the jugular vein; this constraint caused no significant worsening of fit. Full details of the fitting procedure are described in the Appendix.

Table 1 Operational model parameter estimates

Compound	pK_A		τ	
	Platelet	Jugular	Platelet	Jugular
I	7.21 ± 0.29	6.63 ± 0.13	21.34 ± 9.65	4.13 ± 1.66
II	6.69 ± 0.40	6.75 ± 0.15	23.05 ± 10.42	3.31 ± 1.49
III	7.02 ± 0.12	6.31 ± 0.13	2.42 ± 0.31	0.78 ± 0.28
IV	6.69 ± 0.20	6.41 ± 0.26	1.32 ± 0.21	0.44 ± 0.11
V	7.12 ± 0.12	6.80 ± 0.16	3.75 ± 1.42	0.64 ± 0.12
VI	6.94 ± 0.17	6.66 ± 0.45 (6.70 ± 0.05)	0.95 ± 0.27	$0.27 \pm 0.05^*$
VII	6.51 ± 0.25	5.71 ± 0.49 (5.72 ± 0.03)	0.39 ± 0.13	$0.31 \pm 0.08^*$
VIII	(7.05 ± 0.03)	(6.21 ± 0.06)		

Parameter estimates $\pm$ s.e.mean were estimated as described in the Methods section.

Values in parentheses represent pK_B values obtained as described in the text.

Values are the average of 3–6 estimates.

*On many occasions these compounds did not display agonism in this assay.

always less than on occasions when these compounds showed agonism, supporting the assumption of a decreased receptor reserve in these tissues. Compound VIII was 'silent' on all occasions in both the platelet and jugular vein assays and its antagonist effects were also analysed. In these analyses, Compound I was used as the standard agonist. In all four cases, the antagonist compound produced parallel rightward displacement of agonist concentration-effect curves (as assessed by analyses of variance on slopes and asymptotes) and Schild plot slopes were not significantly different from unity. Figure 6 illustrates the Schild plots for each analysis. The pK_B estimates for compounds VI and VII were similar to the pK_A estimates obtained when these compounds behaved as agonists in the jugular vein (Table 1). For compound VIII, pK_B estimates of 7.05 and 6.21 were obtained in the platelet and jugular vein assays respectively. Statistical analysis of the discrepancy is not strictly appropriate since the error estimates were obtained in different ways (see Methods section). However, the estimates are clearly different, the value obtained in the platelet being $0.84 \log_{10}$ units higher than that in the vascular assay. This result is qualitatively consistent with the systematic difference ($0.47 \log_{10}$ units) in the agonist pK_A estimates between the two tissues.

Discussion

The recent availability of a highly potent and selective DP receptor antagonist, BW A868C, has allowed significant advances to be made in the identification and classification of prostaglandin receptors (Giles *et al.*, 1989). Using this antagonist, the DP receptors mediating inhibition of aggregation

in human platelets and relaxation of rabbit vasculature were provisionally concluded to be of the same DP class. However, those studies were limited by the lack of selectivity of the available agonists which meant that a quantitative analysis of BW A868C was not possible in the vascular assay. The natural agonist, PGD_2 , amongst its many actions, elicits vasorelaxation through (presumed) EP_2 receptors; the same is true of BW 245C, the PGD_2 analogue (Giles *et al.*, 1989). Whereas additional actions such as TP receptor activation could be blocked with a suitable antagonist, BM 13.177, the lack of an EP_2 receptor antagonist meant that displacements of agonist-concentration effect curves elicited by BW A868C were confounded by actions through EP_2 receptors.

The series of compounds described in the present paper has allowed this analytical problem to be approached and has enlarged upon the base of information available for the characterization of DP-receptors. In fact, in attempting to perform a quantitative analysis of BW A868C antagonism in the rabbit jugular vein assay using Compound I (572C85) as the agonist a further problem associated with the antagonist was revealed. Despite the specificity of Compound I for DP receptors (evident from the absence of a phase of agonism resistant to BW A868C) it was not possible to analyse concentration-effect curve displacements due to slowing of agonist responses in the presence of BW A868C. This result was suggestive of a 'hemi-equilibrium' state being produced in the jugular vein assay. Hemi-equilibrium is most likely to occur when the antagonist dissociates slowly from the receptor (consistent with the high affinity of BW A868C, $pK_B = 9.26$ (Giles *et al.*, 1989)) and when the agonist requires a high receptor occupancy in order to elicit a response (Rang, 1966). In the platelet assay, which has a higher receptor reserve than

Figure 6 Schild plots showing antagonism of 572C85 (compound I) concentration-effect curves in rabbit jugular vein by (a) compound VI; (b) compound VII; (c) compound VIII and in human platelets by (d) compound VIII. In all cases the slope parameter, n , was not significantly different from unity and the line through each data set was obtained by fitting the data to the appropriate equation (see methods) with n constrained to unity. On this occasion in the platelet assay it was not possible to obtain daily estimates of pK_B therefore all the data were fitted simultaneously to eq. (ii). The pK_B and slope estimates are shown in each panel.

the jugular vein (see Figure 2 and Table 1), such a problem did not occur and the antagonism of Compound I by high concentrations of BW A868C produced no measurable slowing in response. Indeed, the present analysis gave parallel curve displacements and, although the Schild plot slope was slightly greater than unity this, and the pK_B estimate, was indistinguishable from that obtained previously in the platelet assay with BW 245C as the agonist (Giles *et al.*, 1989).

The apparent 'hemi-equilibrium' state in the jugular vein assay was not only produced when Compound I was the agonist employed; the same response slowing was obtained with each of the other agonists. However, when compounds VI-VIII were used as competitive antagonists of compound I, no kinetic problems were revealed and they behaved as simple competitive antagonists in both tissues, presumably because they dissociated from the receptor more quickly than BW A868C. Consistent with this are the estimates of their affinity for the DP receptor, which were 100–1000 fold lower than that of BW A868C (Figure 6 and Table 1).

Therefore, Compound VIII may be preferred to BW A868C for quantitative identification of DP receptors, particularly in assays with a low receptor reserve. It may be wise to recommend BW A868C only for qualitative classification purposes, that is, for discrimination between DP and other types of prostaglandin receptor (its marked selectivity permits this), but not for positive, quantitative definition.

However, despite the problems associated with BW A868C, the tentative conclusion based on its use that the DP receptors in human platelets and rabbit vasculature are the same (Giles *et al.*, 1989) is supported by the combined weight of the affinity and efficacy information obtained here. Figure 5 and analysis (see Appendix) showed that Compounds I-VII demonstrated the same relative affinities and efficacies in the two assays, consistent with similar recognition and transduction of the chemical information in this series by the two receptor populations involved. Similar, that is, but not the same. Evidently, there was a difference between the pK_A estimates for the series between the two tissues (supported in the case of Compounds VI and VII by antagonist affinity (pK_B) estimates) suggesting that the two receptor populations are not identical. However, the difference was systematic and

might be explained by minor differences in micro-environment or amino-acid sequence or difference in, say, the type of orientation of binding sites in two otherwise very similar receptors.

Inevitably, such suggestions raise issues of the definition of a receptor, of what 'receptor similarity' really means and what constitutes hard evidence for classifying receptors as the same in different tissues; these issues have been discussed extensively elsewhere (e.g. Stephenson, 1975; Black *et al.*, 1986). In the 5-HT receptor area it has been useful to consider the chemical similarity or otherwise to the natural agonist of the ligands used for classification of the receptor in question (Leff & Martin, 1986; Leff *et al.*, 1986). The argument is that there may be accessory binding sites within the receptor structure which are not utilised by the natural agonist and which may not be conserved between different tissues. As a result, agonists and antagonists which utilise these sites may express different binding affinities between the tissues, whereas the affinity of the natural agonist does not vary. In the present case we cannot claim that Compounds I-VIII are chemical analogues of PGD_2 and it may be that the systematic differences in affinities of these compounds between the platelet and vascular receptor are a consequence of an accessory binding site(s) difference, in which case the ability of these compounds to detect the sameness of the two receptor populations is limited. On the other hand, it seems reasonable to suggest that the 'fingerprint' of affinity and efficacy information generated for Compounds I-VIII can, as it stands, be used to characterize positively and quantitatively DP receptors in new locations. Whether the systematic differences detected here continue to be manifested between different tissues and whether the use of new compounds can lead to a decrease in such analytical noise and, therefore, more quantitatively precise receptor classification will no doubt become apparent from future studies.

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Appendix: Goodness-of-fit analyses

Analyses of variance

Fit 1 in Table A1 provided an estimate of the error associated with model-fitting. The estimated residual sum of squares (RSS) in Fit 2, which we assume is dominated by inter-preparation variation in receptor reserve, that is, a tissue-dependent contribution to τ , also contains fitting error. Therefore the purely tissue-dependent RSS is $7.98444 - 0.35182$ which is 7.63262 (132 d.f.). Therefore, the mean RSS associated with tissue variation is 0.05782 . This will be considered within the tissues error with which the results of subsequent constraints will be compared.

Analysis of τ

Comparing Fits 2 and 3 shows that the assumption that the τ values for each agonist are the same in each tissue results in an increase in RSS of 2.52906 (7 d.f.) which is equivalent to a mean RSS of 0.36129 . Comparing this error with the within tissues error:

$$F_{7,132} = 0.36129/0.05782 = 6.2486$$

which is highly significant ($P < 0.001$) and indicating, as expected by inspection of the data (see Figure 2), that the efficacy of each agonist differs between the two assays.

Comparing Fit 4 with Fit 2 shows that the assumption that the relative τ values for the series of agonists are the same in each tissue results in an increase in RSS of 0.14231 (6 d.f.) giving a mean RSS of 0.02372 . In this case, comparison with the within tissues error gives:

$$F_{6,132} = 0.02372/0.05782 = 0.4102$$

which is clearly not significant.

Therefore it is reasonable to conclude that the relative efficacies of the compounds are the same in both tissues.

Analysis of pK_A

Comparing Fit 5 with Fit 2 shows that the assumption that the pK_A values for the series of agonists are the same in each tissue results in

an increase in RSS of 0.25473 (7 d.f.) giving a mean increase of 0.03639 . Therefore,

$$F_{7,132} = 0.03639/0.05782 = 0.6294$$

which is not significant, implying that the assumption is valid. However, comparing Fit 6 with Fit 5, which tests whether the assumption of constant increment in the pK_A values for the agonists between tissues results in an improvement in fit over common pK_A values, yields the following: the decrease in RSS is 0.16595 (1 d.f.), therefore,

$$F_{1,132} = 0.16595/0.05782 = 2.8701$$

which approaches significance ($0.05 < P < 0.10$).

Furthermore, it was noticed when comparing Fits 5 and 2 that, in order to accommodate the constraint that pK_A values should be the same, the estimates of τ changed dramatically. This indicated that variation of τ values during model-fitting could absorb some of the error associated with pK_A estimation. This is reasonable since τ , as a parameter in the model, contributes to the location $[(A_{50})]$ of concentration-effect curves as well as K_A . Therefore, Fits 7, 8 and 9 were performed which allowed the same tests as those carried out above to be performed without the possibility that error associated with pK_A estimation could be accommodated by τ error. Now, comparing Fits 7 and 8, constraining pK_A s to be the same in the two tissues resulted in a significant increase in mean RSS.

The difference in RSS was 0.90388 (7 d.f.) giving a mean of 0.12913 . Therefore $F_{7,132} = 0.12913/0.05782 = 2.233$ ($0.025 < P < 0.05$). Furthermore, constraining the difference in pK_A estimates between the two tissues to be constant (comparing Fit 9 with Fit 7) did not result in a significant worsening of fit. The increase in RSS was 0.17809 (6 d.f.) giving a mean of 0.02968 .

$$F_{6,132} = 0.02968/0.05782 = 0.5133$$

which is clearly not significant.

Therefore, from these analyses of variance it appeared sensible to conclude that the τ estimates and the pK_A estimates for the series of agonists were relatively the same in both tissues but differed by constant factors between the two tissues.

Table A1 Goodness-of-fit analyses

Fit	Constraint	Parameters Fitted	Comment	†RSS	d.f.
1	None: a curve fitted to each replicate data set within each treatment (excluding zeros*)	59 pK_A s 59 τ s	Provides estimate of error associated with fitting	0.35182	319
2	A single curve fitted to all replicate data within a treatment	14 pK_A s 14 τ s	Provides estimates of error associated with tissue-dependent receptor-reserve differences	7.98444	451
3	One curve per treatment assuming same τ s between tissues	14 pK_A s 7 τ s		10.51350	458
4	One curve per treatment assuming same relative τ s between tissues	14 pK_A s 7 τ s Q	Q denotes the average factor by which τ values differ between the two tissues	8.12675	457
5	One curve per treatment assuming same pK_A s between tissues	7 pK_A s 14 τ s		8.23917	458
6	One curve per treatment assuming same relative pK_A s between tissues	7 pK_A s 14 τ s C	C denotes the average increment by which pK_A values differ between the two tissues	8.07322	457
7	One curve per treatment assuming τ values fixed to their estimates from Fit 4	14 pK_A s		8.12711	465
8	One curve per treatment assuming τ values fixed to their estimates from Fig 4, and assuming same pK_A s between tissues	7 pK_A s	Fixing the τ values prevents pK_A dependent error being absorbed	9.03099	472
9	As Fit 8 except assuming same relative pK_A s between tissues	7 pK_A s C		8.30520	464

†RSS = residual sum of squares.

*zeros refers to data sets comprised of zero effect values; such results were occasionally obtained for Compounds VI and VII.