Affinities, Selectivities, Potencies, and Intrinsic Activities of Natural and Synthetic Prostanoids Using Endogenous Receptors: Focus on DP Class Prostanoids

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ABSTRACT

The prostanoid receptor-subtype binding affinities, selectivities, potencies, and intrinsic activities of four natural prostanoids and six synthetic DP class prostanoids were determined using binding and functional assays with endogenous receptors. SQ27986 exhibited the highest affinity for the human platelet DP receptor and the best DP receptor selectivity profile. Prostaglandin (PG)D$_2$ was the least DP receptor-selective. The rank order of compound affinities at the DP receptor was SQ27986 (K_i = 10 ± 2 nM) > RS93520 = ZK110841 = BW245C (K_i = 23–26 nM) > ZK118182 (K_i = 50 ± 9 nM) > PGD$_2$ (K_i = 80 ± 5 nM). DP receptor agonists produced cAMP in embryonic bovine tracheal fibroblasts with different potencies (EC$_{50}$ values in nM): ZK118182 (18 ± 6), RS93520 (28 ± 6), SQ27986 (29 ± 7), ZK110841 (31 ± 7), BW245C (53 ± 16), and PGD$_2$ (98 ± 10). BW245C was more efficacious and RS93520 was less efficacious than PGD$_2$. ZK110841 and ZK118182 exhibited a relatively high potency at the adenylyl cyclase-coupled EP$_2$ receptor in human nonpigmented ciliary epithelial cells but were partial agonists. None of the DP class agonists showed any EP$_4$ receptor functional activity in Chinese hamster ovary cells. The DP receptor antagonist BWA868C competitively antagonized the PGD$_2$-induced cAMP accumulation in embryonic bovine tracheal fibroblast cells (pA$_2$ = 7.83 ± 0.08). The dissociation constants for BWA868C antagonizing PGD$_2$-, BW245C-, and ZK118182-induced cAMP production were quite similar (apparent −log K_b = 7.9–8.2, n = 5–9). The pharmacological properties of some natural and numerous DP class synthetic prostanoids have been determined using endogenous receptors.

Prostanoids, including prostaglandins (PGs) such as PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, and PGI$_2$, are endogenous derivatives of arachidonic acid that produce numerous physiological and pathological effects in the mammalian body (see Coleman et al., 1994, for a review). PGD$_2$, which is the natural ligand for the DP receptor, is produced in many organs including brain, lung, skin, and mast cells and has been implicated in the mediation or regulation of body temperature, sleep, hormone secretion, ion transport, pain, and intraocular pressure, in addition to other functions (Leff and Giles, 1992; Coleman et al., 1994; Rangachari et al., 1995). Thus, PGD$_2$ inhibits platelet aggregation (Leff and Giles, 1992; Coleman et al., 1994), induces bronchoconstriction and allergic rhinitis (Hamid-Bloomfield et al., 1990; Coleman et al., 1994), and lowers intraocular pressure (Matsugi et al., 1995).

The effects of PGD$_2$ are mediated by specific membrane-bound DP receptors, which are coupled, via a G$_s$ protein, to

adenylyl cyclase (AC), whose activation results in cAMP production (Trist et al., 1989; Crider et al., 1999). DP receptors in animal and human tissues and cells have been studied pharmacologically using a variety of tissue-based contraction or relaxation functional assays (Coleman et al., 1994; Liu et al., 1996a; Lydford et al., 1996) using some potent agonists such as BW245C (Town et al., 1983; Giles et al., 1989; Leff and Giles, 1992), ZK110841 (Thierauch et al., 1988; Schulz et al., 1990), SQ27986 (Coleman et al., 1994), and a potent antagonist, BWA868C (Giles et al., 1989; Trist et al., 1989; Leff and Giles, 1992; Liu et al., 1996a,b). However, there is a paucity of detailed pharmacological information on the relative receptor binding affinities, receptor-subtype selectivities, and functional in vitro potencies of the latter compounds at the major prostanoid receptor subtypes, especially using radioligand binding and second-messenger assays.

The human and mouse DP receptors were recently cloned (Kiriyama et al., 1997; Wright et al., 1998) and shown to be members of the superfamily of hepta-helical-transmembrane

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ABBREVIATIONS: AC, adenylyl cyclase; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified essential medium; EBTr, embryonic bovine tracheal; IA, intrinsic activity; NPE, nonpigmented ciliary epithelial; pK_b, apparent −log molar antagonist concentration needed to cause dextral shift of agonist concentration-response curve by 2-fold; pA$_2$, −log molar antagonist concentration needed to cause dextral shift of agonist concentration-response curve by 2-fold; PEI, polyethyleneimine; PG, prostaglandin; PGI$_2$, prostacyclin.

domain G protein-coupled receptors. These cloned DP receptors have been expressed in a variety of host cells and preliminary studies conducted to define the pharmacology of these receptors (Kiriyama et al., 1997; Wright et al., 1998). Although these recombinant receptor expression systems represent novel tools to study many facets of drug-receptor interactions and receptor-effector coupling, they do not always faithfully reflect endogenous receptors in cells and tissues (Boddeke et al., 1992; Kenakin, 1996, 1997; Wright et al., 1998). Conversely, receptor heterogeneity and species differences can complicate the interpretation of the results obtained from the natural expression systems. However, on balance the latter systems offer the key advantage of the study of the pharmacology of the receptors in their natural environment, where the stoichiometry of other cellular elements such as G proteins is well defined and controlled. Accordingly, the aims of the present study were to determine the receptor binding affinities, selectivities, and functional potencies and intrinsic activities (IAs) of some natural and numerous synthetic DP class prostanoids using cells and tissues naturally expressing various prostanoid receptors. To our knowledge, this represents the first such comprehensive comparison and definition of the pharmacological properties of these prostanoids using such tissue homogenate- and cell-based assays.

Experimental Procedures

DP Receptor Binding Assay. Human platelets are known to express DP receptors (Cooper and Ahern, 1979). Frozen-thawed human blood platelet membranes (20 mg wet wt. tissue/tube) suspended in 25 mM Tris-HCl, pH 7.4 (containing 138 mM NaCl, 5 mM MgCl$_2$, and 1 mM EDTA) were incubated with 2 to 10 nM [^{3}H]PGD$_2$ in a total volume of 500 μl. Nonspecific binding was defined with 10 μM BWA868C or 10 μM unlabeled PGD$_2$. Both prostanoids yielded the same level of specific binding. The incubations (20 min at 23°C; Cooper and Ahern, 1979) were terminated by rapid vacuum filtration [using Whatman GF/B glass fiber filter previously soaked in 1% polyethyleneimine (PEI) and 0.1% BSA], and the receptor-bound radioactivity was determined by scintillation spectrometry. The data were analyzed by a nonlinear, iterative, curve-fitting program (Bowen and Jerman, 1995; Sharif et al., 1998, 1999) (see later).

EP$_3$ Receptor Binding Assay. The bovine corpus luteum has been shown to express high-affinity [^{3}H]PGE$_2$ binding sites that appear to be of the EP$_3$ subtype (Sharif et al., 1998). Washed total particulate bovine corpus luteum membranes were prepared according to standard homogenization and centrifugation procedures (Sharif et al., 1998) and incubated (16 mg wet wt. tissue/tube final) with [^{3}H]PGE$_2$ (0.9–2 nM) in Krebs' buffer, pH 7.4, for 1 h at 23°C in a total volume of 500 μl. Nonspecific binding was defined with 1 μM unlabeled PGE$_2$. The assays were terminated by vacuum filtration (using Whatman GF/B glass fiber filter previously soaked in 0.3% PEI), and the data were analyzed as described above for DP assays.

FP Receptor Binding Assay. The bovine corpus luteum has been shown to express high-affinity [^{3}H]PGF$_{2\alpha}$ binding sites, in addition to [^{3}H]PGE$_2$ binding, which appear to have pharmacological characteristics of FP receptors (Sharif et al., 1998). Washed total particulate bovine corpus luteum membranes (20 mg wet wt. tissue/tube final) were incubated with [^{3}H]PGF$_{2\alpha}$ (0.9–1.5 nM) in Krebs' buffer, pH 7.4, for 2 h at 23°C in a total volume of 500 μl. Nonspecific binding was defined with 10 μM unlabeled PGF$_{2\alpha}$ or fluprostenol, with both yielding very similar results. The assays were terminated by vacuum filtration (using Whatman GF/B glass fiber filter previously soaked in 0.3% PEI), and the data were analyzed as described earlier for DP assays.

IP and TP Receptor Binding Assays. Human platelets express specific IP (Armstrong et al., 1989) and TP (Ogletree and Allen, 1992) prostanoid receptors. Frozen-thawed human platelet membranes (16 mg wet wt. tissue/tube final) dispersed in 50 mM Tris-HCl containing 10 mM MgSO$_4$, pH 7.4, were incubated with 1 nM [^{3}H]SQ29548 or 3 nM [^{3}H]iloprost to label TP and IP receptors, respectively. Nonspecific binding was defined with 10 μM pinane thromboxane or 10 μM iloprost for TP and IP receptors, respectively. The incubations (60 min at 23°C) were terminated by rapid vacuum filtration (using Whatman GF/B glass fiber filter previously soaked in 0.3% PEI), and the data were analyzed as described above for DP assays.

AC Assays. A number of cell types have been characterized in the literature to show expression of specific endogenous prostanoid receptors functionally coupled to AC (see later). We used these cell types in the current experiments. Thus, embryonic bovine tracheal fibroblasts (EBTrs) for DP receptors (Crider et al., 1999), immortalized human nonpigmented ciliary epithelial (NPE) cells for EP$_2$ receptors (Jumblatt et al., 1994), NCB-20 (mouse neuroblastoma-hamster brain hybridoma) cells for IP receptors (Blair et al., 1980), and Chinese hamster ovary (CHO) cells for EP$_4$ receptors (Crider et al., 2000; all ~90% confluent) were rinsed twice with 0.5 ml of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (DMEM/F-12) at 23°C. The medium was then replaced with 0.5 ml of DMEM/F-12 containing 0.8 mM ascorbate and 1 mM isobutyl methylxanthine. The isobutyl methylxanthine/ascorbate preincubation was carried out for 20 min to inhibit phosphodiesterase activity and thus help accumulate cAMP formed by receptor activation. The cells were then exposed to the various agonists for 15 min at 23°C, a time period over which the cAMP generation was linearly related to time. When the antagonistic effects of BWA868C were investigated, it was incubated with the cells for a total of 60 min at 23°C to permit full equilibrium. The assays were terminated by the addition of 150 μl of ice-cold 0.1 M acetic acid, pH 3.5, and after 5 min, the samples were neutralized with 225 μl of ice-cold 0.1 M sodium acetate (pH 11.5–12.0). The amounts of cAMP produced in the different cell types were determined using a standard cAMP radioimmunoassay kit as directed by the manufacturer and as previously described (Sharif et al., 1997). A standard concentration curve for cAMP was performed, and the unknown samples were run in parallel.

Phosphoinositide Turnover Assays. Swiss 3T3 cells express functional FP receptors but not other prostanoid receptors (Sharif et al., 1998). In the present study, [^{3}H]inositol phosphates ([^{3}H]IPs) produced by agonist-mediated activation of phospholipase C in Swiss 3T3 cells expressing FP receptors were quantified according to previously published procedures (Sharif et al., 1998; Griffin et al., 1999). Briefly, confluent 3T3 cells were exposed to 1.0 to 1.5 μCi of *myo*-[^{3}H]inositol (18.3 Ci/mmol) in 0.5 ml of DMEM for 24 to 30 h at 37°C. Then, cells were rinsed once with DMEM/F-12 containing 10 mM LiCl, and the agonist stimulation experiment was performed in 0.5 ml of the same medium to facilitate accumulation of [^{3}H]IPs. Cells were exposed to the agonist or solvent for 60 min at 37°C (triplicate determinations), followed by aspiration of the medium and the immediate addition of 1 ml of ice-cold 0.1 M formic acid. The plates were kept cold and then frozen. Samples frozen up to 1 week were thawed before chromatographic separation of radiolabeled components. The cell lysates (0.9 ml) were loaded onto columns packed with approximately 1 ml of AG 1-X8 anion exchange resin (formate form). The elution procedure consisted of a wash with 10 ml of H$_2$O, then 8 ml of 50 mM ammonium formate, and finally 4 ml of 1.2 M ammonium formate with 0.1 M formic acid, which was collected in a scintillation vial. To this eluate we added 15 ml of scintillation fluid, and the total [^{3}H]IPs were determined by scintillation counting on a beta counter. Data were analyzed by the sigmoidal fit function of the Origin Scientific Graphics software (Microcal Software, Northampton, MA) to determine agonist potency (EC$_{50}$ value) and IA relative to a full agonist such as fluprostenol or cloprostenol (see later).

Data Analyses. The original data (disintegrations per minute) from the different ligand-binding experiments were analyzed using a

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nonlinear, iterative curve-fitting computer program (Bowen and Jerman, 1995; Sharif et al., 1997, 1999). Additional analyses were performed using the "EBDA" suite of computer programs (McPherson, 1983). The receptor-binding inhibition constants (K_i values) were calculated from IC_{50} values as previously described (Cheng and Prusoff, 1973; Sharif et al., 1998). The Cheng-Prusoff equation is $K_i = IC_{50}/(1 + [L]/K_d)$, where IC_{50} is the compound concentration causing 50% inhibition of the binding, L is the radioligand concentration used in the competition experiments, and K_d is the dissociation constant of the radioligand.

The phosphoinositide turnover functional data were analyzed by the sigmoidal fit function of the Origin Scientific Graphics software (Microcal Software) to determine EC_{50} and IA values. The logistical equation for curve fitting was:

$$\frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2$$

where x_0 is EC_{50} or IC_{50}, p is power, A_1 is minimal Y value, and A_2 is maximal Y value. The apparent IA of the agonists was defined relative to the maximal response to the natural prostanoid or a full agonist (IA set to 1.0 for full agonist; i.e., 100% activity) in each receptor system. Thus, for the DP receptor, the maximal response to PGD_2 was set to 1.0 and the responses of other prostanoids were defined relative to this. Data from functional assays in which various antagonist concentrations were used against a single agonist concentration were analyzed with the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Schild analyses of BWA868C-induced antagonism of PGD_2-induced cAMP production in EBTr cells were performed as previously described (Arunlakshana and Schild, 1959) and as recently conducted (Wiernas et al., 1997; Griffin et al., 1999). In additional experiments, single antagonist concentration-induced dextral shifts of agonist-induced cAMP production curves were analyzed

according to Furchgott (1972) where apparent K_b = (antagonist concentration)/[(agonist EC_{50} in the presence of antagonist/agonist EC_{50} in the absence of the antagonist) − 1]. A one-way ANOVA and t test were used to determine possible statistical significance between the data sets.

Materials. Swiss albino mouse 3T3 fibroblasts, CHO-K1 cells, and EBTr cells were obtained from American Type Culture Collection (Rockville, MD). Immortalized NPE cells were the generous gifts of Dr. M. Coca-Prados (Yale University, New Haven, CT). Out-of-date human platelet-enriched plasma was obtained from a local blood bank. Bovine corpora lutea were obtained from Pel-Freez (Rogers, AR). Tissue culture and other reagents, including DMEM, DMEM/F-12, glutamine, gentamycin, trypsin/EDTA, PBS without Ca^{2+} or Mg^{2+}, Hanks' balanced salt solution, and HEPES were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum (Hyclone, Logan, UT) was heat-inactivated at 56°C for 30 min and stored at −20°C. EDTA (disodium salt), Tris base, BSA, digitonin, formic acid, ammonium formate, LiCl, and polyethylenimine were obtained from Sigma Chemical Co. (St. Louis, MO). EGTA was obtained from Fluka BioChemika (Buchs, Switzerland). *myo*-[^{3}H]inositol (18.3 Ci/mmol) and [^{3}H]iloprost (12.7 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). [^{3}H]PGD$_2$ (115 Ci/mmol), [^{3}H]PGF$_{2\alpha}$ (170 Ci/mmol), [^{3}H]PGE$_2$ (171 Ci/mmol), and [^{3}H]SQ29548 (50.4 Ci/mmol) were obtained from New England Nuclear (Boston, MA). cAMP radioimmunoassay kits were purchased from PerSeptive Diagnostics (Cambridge, MA). AG 1-X8 anion exchange resin was obtained from Bio-Rad (Hercules, CA). Ecolume scintillation fluid was purchased from ICN Biomedicals (Costa Mesa, CA). All prostanoids were purchased from Cayman Chemical Co. (Ann Arbor, MI) or synthesized at Alcon or by contract using published methods, except as follows: ZK110841 and ZK118182 were the generous gifts of Schering AG (Berlin and Bergkamen, Germany),

TABLE 1
Receptor binding affinities of some natural prostanoids at the major prostanoid receptors

Values are mean ± S.E. for three to five experiments except for values for PGD_2, which were from 63 experiments. Selectivity parameters (in parentheses) refer to the ratio of affinities between the nonpreferred receptor and the preferred receptor known for the particular prostanoid. Thus, for example, the preferred receptor for PGD_2 is the DP receptor; for PGE_2, it is the EP_3 receptor; for $PGF_{2\alpha}$, it is the FP receptor; and for PGI_2, it is the IP receptor. The Hill coefficients for the compounds competing at the DP receptor were 0.96 ± 0.03 for PGD_2, 1.1 ± 0.04 for PGE_2, and 0.94 ± 0.1 for $PGF_{2\alpha}$.

| Compound | Receptor Binding Affinity (K_i) and Selectivity Parameters | | | | |
	DP Receptors (human)	EP₃ Receptors (bovine)	FP Receptors (bovine)	IP Receptors (human)	TP Receptors (human)
			nM		
PGD₂	80 ± 5	1100 ± 150 (14×)	2500 ± 760 (31×)	>148,000 (1850×)	>35,600 (445×)
PGE₂	11,000 ± 2100 (3666×)	3 ± 0.2	3358 ± 719 (1119×)	>4000[a] (1333×)	N.D.
PGF₂α	18,000 ± 6400 (153×)	24 ± 9 (−5×)	118 ± 7	>50,000 (424×)	>190,000 (1610×)
PGI₂	3537[b] (3×)	5374 ± 1395 (4×)	86,000 ± 29,000 (62×)	1390 ± 724	N.D.

N.D., not determined.
[a] From McDermot et al., 1981.
[b] From Shimizu et al., 1982.

TABLE 2
Receptor binding affinities and selectivities of PGD_2 and some synthetic DP class prostanoids

Values are mean ± S.E. from three to five experiments except for values for PGD_2, which were from 63 experiments. Values in parentheses denote the selectivity of the compounds at the particular receptor subtype relative to DP receptors. The Hill coefficients for the compounds competing at the DP receptors were 0.96 ± 0.03 for PGD_2, 0.96 ± 0.13 for BW245C, 0.96 ± 0.1 for ZK110841, 0.99 ± 0.1 for ZK118182, 1.3 ± 0.3 for SQ27986, 0.97 0.13 for RS93520, and 1.23 ± 0.18 for BWA868C. For reference, the affinities of other selective compounds for their respective receptors were as follows: EP₃ selective: sulprostone EP₃, K_i = 11 nM; FP selective: cloprostenol FP, K_i = 34 nM; IP selective: iloprost IP, K_i = 4 nM; and TP selective: SQ29548 TP, K_i = 13 nM.

| Compound | Receptor Binding Affinity (K_i) and Selectivity Parameters | | | | |
	DP Receptors (human)	EP₃ Receptors (bovine)	FP Receptors (bovine)	IP Receptors (human)	TP Receptors (human)
			nM		
PGD₂	80 ± 5	1100 ± 150 (14×)	2500 ± 760 (31×)	>148,000 (1850×)	>35,600 (445×)
BW245C	25 ± 3	143,000 ± 66,000 (5720×)	>100,000 (4000×)	31,800 ± 13,400 (1272×)	141,000 ± 6000 (5640×)
ZK110841	26 ± 4	2900 ± 150 (112×)	20,000 ± 2000 (769×)	4000 ± 50 (154×)	22,500 ± 1000 (865×)
ZK118182	50 ± 9	8700 ± 2500 (174×)	69,000 ± 19,000 (1380×)	45,900 ± 9600 (900×)	94,400 ± 2300 (1888×)
SQ27986	10 ± 2	>77,000 (7700×)	>100,000 (10,000×)	54,800 ± 7700 (5480×)	92,000 ± 8000 (9200×)
RS93520	33 ± 8	12,900 ± 1900 (391×)	>18,000 (545×)	40,500 ± 5300 (1227×)	75,000 ± 2000 (2273×)
BWA868C	23 ± 5	>25,300 nM (1100×)	86,000 ± 12,000 (3739×)	>62,500 (2717×)	>16,600 (445×)
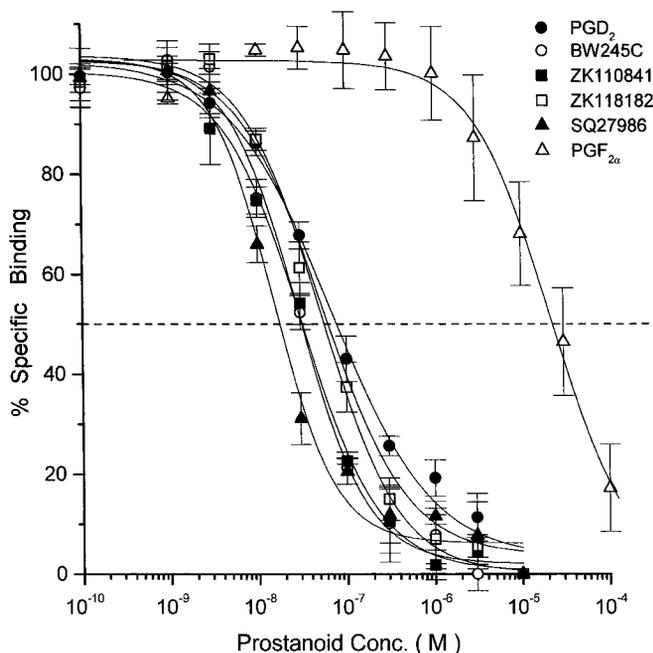
Fig. 1. Concentration-inhibition curves for various prostanoids competing for specific [³H]PGD₂ binding to DP receptors on human platelet membranes. Data are mean ± S.E. from a representative experiment; composite data from numerous such experiments are shown in Tables 1 and 2.

BWA868C and BW245C were the generous gifts of Glaxo-Wellcome (Stevenage, UK), and RS93520 was the generous gift of Hoffman-La Roche (Basel, Switzerland). The chemical structures of the key prostanoids used in these studies were (5Z,13E)-(9R,11R,15S)-9-chloro-15-cyclohexyl-11,15-dihydroxy-3-oxa-16,17,18,19,20-pentanor-5,13-prostadienoic acid (ZK118182), (5Z,13E)-(9R,11R,15S)-9-chlor-15-cyclohexyl-11,15-dihydroxy-16,17,18,19,20-pentanor-5,13-prostadienoic acid (ZK110841), (C3′S,1R,2R,3S,6R)-2-C3′-cyclohexyl-3′hydroxyprop-1-ynyl)-3-hydroxybicyclo[4.2.0]oct-7-ylidene)butyrate (RS-93520), [1S-[1B,2B(5Z), 3A(1E,3S),4B]]7-[3-(3-cyclohexyl-3-hydroxy-1-propenyl)-7-oxabi-cyclo-[2.2.1]hept-2-yl]5-heptenoic acid (SQ27986), 5-(6-cyclohexylhexyl)-1-(3-cyclohexyl-3-hydroxypropylhydantoin) (cloprostenol), 16-m-chlorophenoxy tetranor PGF₂α (BW245C), (5Z,13E)-(9S,11R,15S)-9,11,15-trihydroxy-5,13-prostadienoic acid (fluprostenol), and (±)-3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)hydantoin (BWA868C). Other chemical descriptions of prostanoids can be found in the review by Coleman et al. (1994).

Results

In competition assays, the natural prostanoids exhibited comparatively low affinities and low receptor-subtype selectivities (Table 1). Of the synthetic prostanoid agonists tested, SQ27986 exhibited the highest affinity at the DP receptor and the best receptor selectivity profile, being >5000- to 10,000-fold selective for the DP receptor than at EP₃, FP, IP, and TP receptors (Table 2). The rank order of compound affinities at the DP receptor was SQ27986 (K_i = 10 ± 2 nM) > RS93520 (K_i = 23 ± 5 nM) > ZK110841 (K_i = 26 ± 4 nM) = BW245C (K_i = 25 ± 3 nM) > ZK118182 (K_i = 50 ± 9 nM) > PGD₂ (K_i = 80 ± 5 nM) (Fig. 1; Table 2). PGD₂ itself was the least DP receptor-selective (Table 2).

In functional second-messenger assays, the respective potencies of the DP receptor agonists at stimulating cAMP production via DP receptors in EBTr cells were ZK118182 (EC₅₀ = 18 ± 6 nM) > RS93520 (EC₅₀ = 28 ± 6 nM) = SQ27986 (EC₅₀ = 29 ± 7 nM) = ZK110841 (EC₅₀ = 31 ± 7 nM) > BW245C (EC₅₀ = 53 ± 16 nM) > PGD₂ (EC₅₀ = 98 ± 10 nM) ≫ PGE₂ (EC₅₀ > 1 μM) (Fig. 2; Tables 3 and 4). In comparison with PGD₂ (IA = 1.0), BW245C (IA = 1.17 ± 0.02; P < .01) was more efficacious and RS93520 (IA = 0.75 ± .04; P < .05) was less efficacious than PGD₂ at the DP receptor (Table 4). Some of the DP receptor agonists tested exhibited relatively high potency at the AC-coupled EP₂ receptor (ZK110841 EC₅₀ = 187 nM; ZK118182 EC₅₀ = 711 nM), but all had low relative IA values (IA = 0.11–0.49; Table 4). Even though PGE₂ (EC₅₀ = 57 nM), iloprost (EC₅₀ = 75 nM), and fluprostenol (EC₅₀ = 4 nM) were potent agonists in the EP₄, IP, and FP receptor functional assays, respectively (see legend for Table 4), none of the selective DP receptor agonists tested showed activity in any of these assays (Table 4). In the latter respect, it was of interest to note that EBTr cells minimally responded to PGE₂ or PGI₂, indicating the absence of EP class and IP receptors. Likewise, NPE cells did not respond to PGD₂, PGF₂α, and PGI₂, indicating a lack of DP, FP, or IP receptors. NCB-20 cells did not respond to PGD₂ and PGF₂α and had a weak response to PGE₂, indicating the lack of DP, EP class, or FP receptors. CHO cells did not respond much to PGD₂, PGF₂α, and PGI₂, indicating the lack of endogenous DP, FP, or IP receptors.
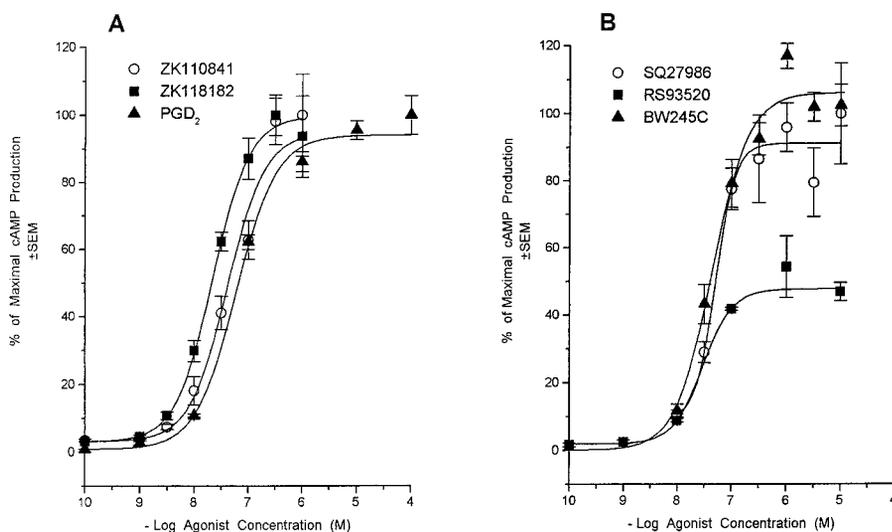
Fig. 2. Concentration-response curves for various prostanoids stimulating cAMP production in EBTr cells expressing constitutive DP receptors. Data are mean ± S.E. from a representative experiment; composite data from numerous such experiments are shown in Tables 3 and 4.

TABLE 3

Potencies of some key natural prostanoids at the major prostanoid receptors

Data are mean ± S.E. from three to six independent experiments. The apparent IA of the agonists was defined relative to that of the maximal response (which was set at 1.0 for full agonist) to the natural prostanoid or a full agonist in each receptor system.

Compound	Relative Potencies (EC_{50}) and Intrinsic Activities (IA) at Prostanoid Receptor Subtypes				
	DP Receptors (AC-coupled) (bovine)	EP$_2$ Receptors (AC-coupled) (human)	EP$_4$ Receptors (AC-coupled) (hamster)	IP Receptors (AC-coupled) (hamster/mouse)	FP Receptors (PLC-coupled) (mouse)
	nM				
PGD$_2$	98 ± 10 (IA = 1 ± 0.04)	>10,000	>10,000	>10,000	155 ± 30 (IA = 0.49 ± 0.1)[b]
PGE$_2$	>1000	38 ± 4 (IA = 1 ± 0.06)	57 ± 15 (IA = 1 ± 0.05)	3000 ± 700 (IA = 1 ± 0.04)	2600 ± 560 (IA = 0.59 ± 0.08)[b]
PGF$_{2\alpha}$	>10,000	>10,000	>10,000	>10,000	20 ± 3 (IA = 1)
PGI$_2$	>10,000	>10,000	435–3280[a] and >10,000	4 ± 0.7 (IA = 1 ± 0.05)	>10,000

[a] From Coleman et al., 1995.
[b] Statistically significant compared with IA of full agonist (e.g., PGF$_{2\alpha}$ or fluprostenol) in this system, $P < .01$.

TABLE 4

Potencies and intrinsic activities of DP class prostanoids at major prostanoid receptors

Data are mean ± S.E. from three to six experiments, each performed in duplicate. Values in parentheses denote the relative IA of the compounds at the particular receptor subtype relative to IA of the natural endogenous prostanoid for that receptor or other full agonist (for which IA was set at 1.0). For reference, the potencies of other key compounds for their respective receptors were as follows: IP selective: iloprost, $EC_{50} = 75$ nM; and FP selective: fluprostenol, $EC_{50} = 4$ nM.

Compound	Relative Potencies (EC_{50}) and Intrinsic Activities (IA) at Prostanoid Receptor Subtypes				
	DP Receptors (AC-coupled) (bovine)	EP$_2$ Receptors (AC-coupled) (human)	EP$_4$ Receptors (AC-coupled) (hamster)	IP Receptors (AC-coupled) (hamster/mouse)	FP Receptors (PLC-coupled) (mouse)
	nM				
PGD$_2$	98 ± 10 (IA = 1 ± 0.04)	>10,000	>10,000	>10,000	155 ± 30 (IA = 0.49 ± 0.08)***
BW245C	53 ± 16 (IA = 1.17 ± 0.02)**	6380 ± 3500 (IA = 0.18 ± 0.05)***	>10,000	>10,000	Inactive up to 10 μM
ZK110841	31 ± 7 (IA = 1 ± 0.04)	187 ± 31 (IA = 0.49 ± 0.06)***	N.D.	>8000	Inactive up to 10 μM
ZK118182	18 ± 6 (IA = 0.97 ± 0.08)	711 ± 168 (IA = 0.49 ± 0.07)***	>10,000	7270 ± 450 (IA = 0.88 ± 0.02)	Inactive up to 10 μM
SQ27986	29 ± 7 (IA = 1 ± 0.04)	2120 ± 628 (IA = 0.11 ± 0.05)***	>10,000	>10,000	Inactive up to 10 μM
RS93520	28 ± 6 (IA = 0.75 ± 0.04)*	>10,000	>10,000	>10,000	Inactive up to 10 μM

N.D., not determined.
Statistically significant relative to intrinsic activity of the full agonist prostanoid, * $P < .05$, ** $P < .01$, *** $P < .001$.

Mouse Swiss 3T3 cells contain an FP receptor with which PGD$_2$ and PGE$_2$ cross-react based on their relatively low potencies and IAs. We looked for TP and IP receptors in Swiss 3T3 cells but could not find them (data not shown).

The DP receptor antagonist BWA868C displayed a high DP receptor binding affinity ($K_i = 23 ± 5$ nM; Fig. 1, Table 2) and a high functional potency ($IC_{50} = 43.3 ± 11.5$ nM, $-\log K_i =$ 8.1, versus BW245C; $IC_{50} = 43.6 ± 3.6$ nM, $-\log K_i = 7.7$, versus PGD$_2$; $n = 4$ for each) in blocking PGD$_2$- and BW245C-induced stimulation of AC activity in EBTr cells expressing DP receptors (e.g., Fig. 3). However, BWA868C was inactive in antagonizing functional responses at EP$_2$ and FP receptors (data not shown). In further functional antagonist studies, BWA868C dextrally shifted the concentration-response curves to PGD$_2$ without apparently suppressing the maximal agonist-stimulated responses (Fig. 4A); thus, BWA868C behaved as a competitive antagonist of the PGD$_2$-induced AMP accumulation in EBTr cells [pA$_2$ ($-\log$ molar antagonist concentration needed to cause dextral shift of agonist concentration-response curve by 2-fold) = 7.83 ± 0.08, slope = 1.29 ± 0.04; Fig. 4, A and B). Similar experiments with two other agonists (BW245C and ZK118182) also yielded similar pA$_2$ values (7.9–8.0) for the antagonism of the agonist-induced cAMP production by BWA868C (e.g., Fig. 5). Furthermore, in experiments in which single concen-

trations of BWA868C were used to shift the concentration-response curves of three different DP class agonists, the following dissociation constants ($-\log K_b$; pK$_b$ values) were obtained: pK$_b$ = 8.03 ± 0.15 ($n = 7$) against PGD$_2$, pK$_b$ = 8.21 ± 0.08 ($n = 9$) against ZK118182, and pK$_b$ = 7.9 ± 0.24 ($n = 5$) against BW245C.

Discussion

Endogenous prostanoids are relatively labile and notoriously nonselective with respect to interacting with the various prostanoid receptor subtypes as shown in isolated tissue preparations (Coleman et al., 1994). For instance, even though PGD$_2$ preferentially interacts with the DP receptor, it also has a relatively high potency at FP (Coleman et al., 1994; Sharif et al., 1998), EP$_2$ (Jumblatt et al., 1994), and TP (Hamid-Bloomfield et al., 1990) receptors. Furthermore, PGE$_2$ and PGF$_{2\alpha}$ had a relatively high affinity for the recombinant human DP receptor expressed in HEK 293 cells (Wright et al., 1998); PGF$_{2\alpha}$ binds to EP class receptors (Coleman et al., 1994; Sharif et al., 1998; Table 1), and PGE$_2$ does not discriminate well between the EP receptor subtypes (Kiriyama et al., 1997). Presently, we confirmed this lack of selectivity of PGD$_2$ and PGF$_{2\alpha}$ using ligand binding techniques and using constitutive receptors in certain tissues
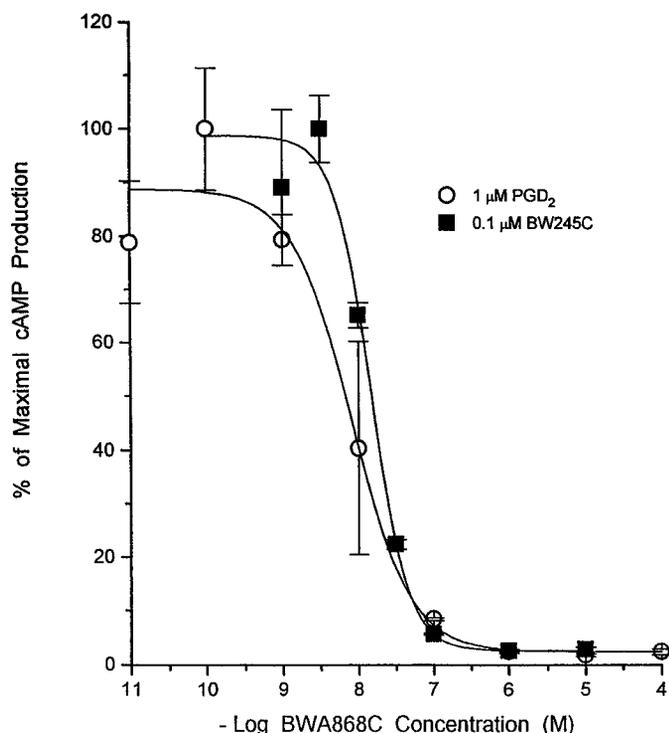
Fig. 3. Inhibition of PGD_2- and BW245C-induced cAMP production in EBTr cells by various concentrations of BWA868C. Data are mean ± S.E. from a representative experiment; composite data from numerous such experiments are shown in *Results*.

known to express these receptors (Table 1). As a result of the problems alluded to earlier with the natural prostanoids, various synthetic prostanoids with agonist and antagonist properties have become available in recent years that appear to show some receptor selectivity (Coleman et al., 1994, 1995). However, in most cases, the pharmacological effects of these compounds have been determined in a limited number of in vitro systems, mostly tissue contraction and/or relaxation assays (e.g., Giles et al., 1989; Leff and Giles, 1992; Lydford et al., 1996) or neurotransmitter release assays (e.g., Ohia and Jumblatt, 1990), and against a limited number of prostanoid receptor subtypes. Ligand binding and second-messenger assays have not been extensively used so far for the determination of the receptor affinities, relative selectivities, and functional potencies of prostanoids in a parallel comparative manner using endogenous receptors along the lines of our investigations. In the present study, we show that the natural prostanoids have affinity for and exhibit IA at their preferred receptors but also at other classes of prostanoid receptors (Tables 1 and 3); hence, they are somewhat nonselective. In contrast, the synthetic DP receptor agonists (e.g., SQ27986, ZK118182, RS93520) and the DP receptor antagonist BWA868C showed a considerably greater degree of selectivity for DP receptors compared with the EP_3, FP, IP, and TP receptors (Table 2), with SQ27986 being the most DP receptor-selective and the one with the highest apparent DP receptor affinity (Table 2). Interestingly, however, BW245C and ZK110841 also exhibited high affinities for the recombi-
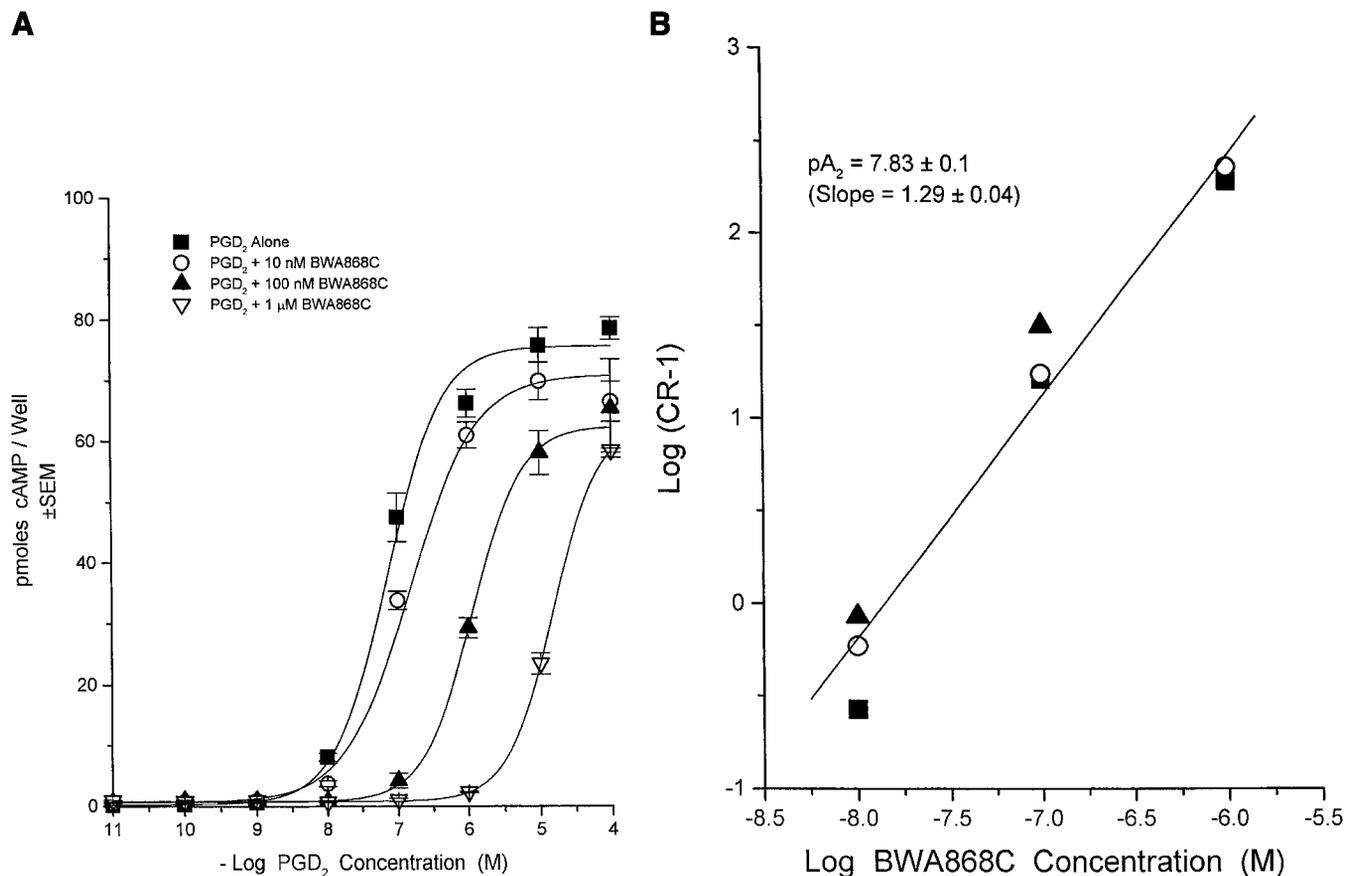

Fig. 4. Schild analysis of inhibition of PGD_2-induced cAMP production in EBTr cells by BWA868C. A, concentration-response curves for PGD_2 in the presence of various concentrations of BWA868C from a representative experiment. B, Schild plots of data from three such experiments denoted by the different symbols.
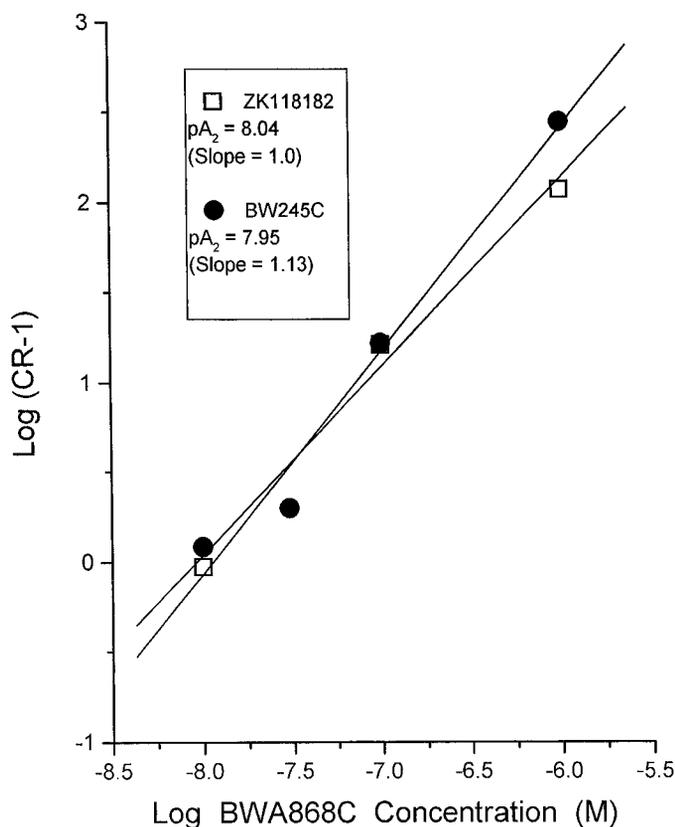
Fig. 5. Schild plots for BWA868C inhibiting BW245C- and ZK118182-induced cAMP in EBTr cells from a representative experiment. Experiments were performed and analyzed as described in the legend to Fig. 4.

nant human EP_2 and EP_4 receptors (Wright et al., 1998), thus making them somewhat less DP receptor-selective. Unfortunately, Wright et al. (1998) and Kiriyama et al. (1997) did not test SQ27986, ZK118182, or RS93520 in their recombinant DP receptor expression systems, so we cannot compare our results for these compounds with theirs. It was noteworthy that although the Hill coefficients of the competition curves for the majority of the compounds we evaluated at the DP receptor in the human platelets were close to unity (Tables 1 and 2; Fig. 1), most compounds (including the DP receptor antagonist BWA868C) showed Hill coefficients of significantly less than unity at the cloned mouse and human DP receptors (Kiriyama et al., 1997; Wright et al., 1998). These differences may be related to the expression levels of the receptor protein in these recombinant systems versus the naturally expressed DP receptors in human platelets used in our studies. Even though different tissues and species were represented among the data sets obtained in our studies (Tables 1–4), the compounds were tested in parallel in these systems, and thus the compounds could be rank ordered within and across the various receptor systems. The affinities and potencies, although designated with certain numerical values, are still relative values and should not be taken as absolute values in view of the earlier statements.

In functional second-messenger studies, although PGD_2 was a full agonist of moderate potency at the DP receptor in EBTr cells, it also exhibited relatively high potency at the FP receptor while being a partial agonist in this system (Table 3). Likewise, PGE_2 exhibited nanomolar potency at the EP_2 and EP_4 receptors (Table 3), again underscoring the relative

lack of selectivity of these natural prostanoids; thus, there continues to be a need for more selective agents for these receptor subtypes. $PGF_{2\alpha}$ appeared to be somewhat FP receptor-selective in our current functional assays, although it had a higher affinity at the EP_3 receptor than at the FP receptor (Table 1). The significance of this finding is not clear, especially because in the recombinant mouse receptor expression system, $PGF_{2\alpha}$ had a higher affinity at the FP receptor than at the EP_3 receptor (Kiriyama et al., 1997), but this perhaps further underscores the possible species differences encountered in the prostanoid field (Ogletree and Allen, 1992; Coleman et al., 1994). In contrast to PGD_2, the other DP class synthetic prostanoids (i.e., SQ27986, BW245C, ZK118182, and ZK110841) were potent DP receptor agonists with EC_{50} values in the range of 18 to 53 nM and all were full agonists, with BW245C being a more efficacious agonist than all the compounds studied (Table 4). The latter confirmed our earlier findings (Crider et al., 1999). Even though PGD_2 itself did not appear to stimulate the EP_2 receptor, the other DP receptor agonists all partially activated EP_2 receptors in the NPE cells with varying degrees of IAs (IA = 0.11–0.49) (Table 4). The data for BW245C confirm similar previous observations (Giles et al., 1989). PGD_2, ZK118182, and RS93520 had little or no functional activity at the IP receptors. From these functional studies, SQ27986, BW245C, and RS93520 appeared to be the most DP receptor-selective prostanoids. However, RS93520 was a partial agonist at the DP receptor, whereas BW245C apparently exhibited greater IA than the other two compounds. Taken together, the high-affinity, relative receptor selectivity, functional potency, and full agonist properties of SQ27986 appear to suggest the use of this prostanoid to characterize DP receptors in cells and tissues in future studies.

In terms of the antagonism of the DP receptor, BWA868C inhibited PGD_2- and BW245C-induced cAMP production with nanomolar potencies (pK_i, pK_b, and pA_2), exhibiting competitive antagonist characteristics when tested against PGD_2, ZK118182, and BW245C in the EBTr cells in the present study (Figs. 4 and 5; *Results*). These antagonist data were comparable with those previously reported for the ability of BWA868C to antagonize DP receptor-mediated functional responses in human platelets (Trist et al., 1989), rabbit jugular vein (Giles et al., 1989), human myometrium (Fernandes and Crankshaw, 1995), various canine blood vessels (Liu et al., 1996a), and rabbit saphenous vein (Lydford et al., 1996). The competitive antagonism by BWA868C observed against PGD_2, BW245C, and ZK118182 in the current study underscored the agonist-independent nature of the blockade of the DP receptor on EBTr cells in our studies. Curiously, although BWA868C behaved as a bona fide antagonist in our studies, antagonizing the functional responses in an agonist-independent manner (see *Results*), it has been reported to exhibit potent agonist activity and high IA at the recombinant human DP receptor (EC_{50} = 7.5 nM, IA = 0.68 relative to PGD_2; Wright et al., 1998) and as a partial agonist stimulating Cl^- secretion in the dog tracheal epithelium (Liu et al., 1996b). Such differences in the agonist and antagonist nature of BWA868C probably reflect differences in the DP receptor reserves in the systems, and perhaps also species differences. These types of results illustrate the need for caution in ascribing pharmacological properties of compounds without carefully defining the system under study.

In conclusion, our survey of four natural prostanoids and six synthetic DP class prostanoids, evaluated in parallel in a number of receptor binding and second-messenger assays, showed that the natural prostanoids for the most part are somewhat nonselective agents. The synthetic DP class receptor compounds like SQ27986, RS93520, and ZK118182 were high-affinity and high-potency agonists, with a high selectivity for the DP receptor. In the EBTr cell system, BWA868C was a potent DP receptor antagonist when tested against three different agonists, and it exhibited a high affinity at the platelet DP receptor. We believe data of this type should prove useful for future pharmacological studies involving the physiological or pathological roles of the DP prostanoid receptor.

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