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Prostaglandin DP receptors positively coupled to adenylyl cyclase in embryonic bovine tracheal (EBTr) cells: pharmacological characterization using agonists and antagonists

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1 Various prostaglandin agonists representing various classes of receptor subtypes were evaluated for their ability to stimulate adenylyl cyclase *via* the endogenous DP receptor in embryonic bovine tracheal (EBTr) cells. Two antagonists were used to block the agonist-induced cyclic AMP production.

2 ZK118182 ($EC_{50}=16\pm4$ nM), RS-93520 ($EC_{50}=23\pm4$ nM), SQ27986 ($EC_{50}=33\pm9$ nM), ZK110841 ($EC_{50}=33\pm5$ nM), BW245C ($EC_{50}=59\pm19$ nM) and PGD_2 ($EC_{50}=101\pm10$ nM) ($n=4-70$) were the most potent agonists. Whilst most compounds were full agonists ($E_{max}=100\%$ relative to PGD_2), BW245C was significantly more efficacious than PGD_2 ($E_{max}=121\pm3\%$; $P<0.001$) and RS-93520 appeared to be a partial agonist ($E_{max}=64\pm9\%$; $P<0.001$).

3 Agonists from the EP (e.g. enprostil; misoprostol; butaprost), FP (e.g. cloprostenol; fluprostenol; PHXA85), IP (iloprost; PGI_2) and TP (U46619) prostanoid receptor classes were weak agonists or inactive in the EBTr cell assay system.

4 The DP-receptor antagonist, BWA868C, showed a competitive antagonist profile with pA_2 values of 8.00 ± 0.02 and 8.14 ± 0.13 in Schild analyses with two structurally different agonists, BW245C and ZK118182, respectively ($n=3$). AH6809, another purported DP-receptor antagonist, weakly inhibited PGD_2- and ZK118182-induced cyclic AMP production (K_is $=808\pm193$ nM and 782 ± 178 nM, respectively).

5 The current studies have characterized the DP receptor positively coupled to adenylyl cyclase in EBTr cells using a wide range of agonist and antagonist prostaglandins. These data support the utility of the EBTr cell line as a useful tool for the evaluation of DP receptor agonists and antagonists and for profiling other classes of prostaglandins.

Keywords: Prostaglandin; DP receptor; EBTr cells; BW245C; BWA868C; AH6809; ZK118182; RS93520; cyclic AMP; adenylyl cyclase

Abbreviations: EC_{50}, concentration of agonist required to produce 50% of the maximal response (potency); E_{max}, per cent of the maximal response (intrinsic activity; efficacy); IC_{50}, concentration of inhibitor required to produce 50% of the maximal inhibition; K_i, concentration of inhibitor required to produce 50% of the maximal inhibition at equilibrium; pA_2, equilibrium dissociation constant of the antagonist; RIA, radioimmunoassay

Introduction

The classification for prostanoid receptors that bind the endogenous prostaglandins, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 are designated DP, EP (with subtypes EP_1, EP_2, EP_3 and EP_4), FP, IP, and TP, respectively (Kennedy *et al*., 1982; Coleman *et al*., 1984; 1994; Alexander & Peters, 1998). These membrane-bound receptors belong to the superfamily of G-protein-coupled receptors containing seven membrane spanning regions (Narumiya, 1994). EP_1, FP, and TP receptors are coupled to phospholipase C *via* either G_q or $G_{q/11}$ which stimulates inositol trisphosphate formation and intracellular Ca^{2+}-mobilization (Coleman *et al*., 1994; Narumiya, 1994). DP, EP_2, EP_4 and IP receptors are positively coupled *via* the G_s protein to adenylyl cyclase which catalyzes the hydrolysis reaction of ATP to the second messenger cyclic AMP (Sugama *et al*., 1989; Namba *et al*., 1994; Boie *et al*., 1995). Although the DP prostanoid receptor has been identified in platelets, smooth muscle cells from various tissues (Coleman *et al*., 1994), neutrophils (Darius *et al*., 1994; Wheeldon & Verdey, 1993), and retina (Boie *et al*., 1995), it has been studied less than other prostanoid receptors due to its low abundance. The DP receptor is thought to be phylogenetically most closely related to the EP_2 and IP receptors (Boie *et al*., 1995). Recently, the DP receptor has been cloned, expressed in transfected cells, and has undergone limited pharmacologic characterization (Hirata *et al*., 1994; Boie *et al*., 1995; Kiriyama *et al*., 1997). As is the case in the EP prostanoid class, there is evidence in the literature to suggest the possibility of subtypes of the DP receptor in human myometrium (Fernandes & Crankshaw, 1995) and canine colonic epithelium (Rangachari *et al*., 1995), for example.

Agents from the DP receptor class produce a wide variety of biological effects (see Andersen & Ramwell, 1974; Negishi *et al*., 1993). Prostaglandin D_2, for example, causes vascular relaxation (Leff & Giles, 1992; Lydford *et al*., 1996) and the inhibition of platelet aggregation (Ito *et al*., 1989; 1990). DP receptor agonists stimulate chloride secretion in canine tracheal epithelial cells (Liu *et al*., 1996a). In addition, bronchoconstriction is caused by DP receptor activation and this effect appears to be involved in asthmatic complications (Johnston *et al*., 1995). Agents from this prostanoid receptor class have also been shown to lower intraocular pressure (IOP)

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(Matsugi *et al*., 1995; Camras & Alm, 1997). The selective DP receptor antagonist, BWA868C, is known to inhibit adenylyl cyclase in human platelets stimulated by PGD_2 (Trist *et al*., 1989) and a less-selective PG receptor antagonist, AH6809, inhibits the anti-aggregation effect of PGD_2 on human platelets (Keery & Lumley, 1988). Some relatively selective DP-receptor agonists include ZK118182 ((5Z,13E)-(9R, 11R, 15S) 0- 9- chloro-15- cyclohexyl -11, 15- dihydroxy- 3- oxo - 16, 17 ,18, 19, 20 - pentanor - 5 ,13 - prostadienoic acid)), ZK110841 ((5Z,13E)-9R,11R,15S)-9b-chlor-15-cyclohexyl-11, 15 -dihydroxy - 16, 17, 18, 19, 20 - pentanor-5, 13 - prostadienoic acid)), SQ27986 ([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)), BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl-hydantoin)) and RS-93520 ((C3′S,1R,2R, 3S, 6R) -2- C3′-cyclohexyl-3′hydroxyprop-1-ynyl)-3-hydroxybicyclo[4.2.0]oct-7-ylidene)butyrate)) (see Coleman *et al*., 1994; Alexander & Peters, 1998).

In preliminary studies, bovine embryonic tracheal (EBTr) cells produced cyclic AMP when stimulated with PGD_2 (Sugama *et al*., 1989) and AH6809 antagonized these effects (Ito *et al*., 1990). However, these constituted limited pharmacological studies on the DP receptor in this cell-line. Therefore, the aims of the present studies were to evaluate several prostaglandin agonists from various receptor classes, as well as to study the effects of the antagonists BWA868C and AH6809, in the EBTr cells using the generation of cyclic AMP as an index of receptor activation in order to better characterize the DP receptor endogenously expressed in these cells.

Methods

Cell culture and cyclic AMP generation

EBTr cells of passages 29 – 34 were seeded into 48 well culture plates at a density of approximately 4×10^3 cells per well. The cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air and fed semi-weekly with Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/l glucose and supplemented with 2 mM glutamine, 10 mg ml^{-1} gentamicin, and 10% foetal bovine serum. Upon reaching confluence, the cells were rinsed twice with 0.5 ml of DMEM/F-12. The sample wells were then pre-incubated for 20 min with 0.8 mM ascorbate and 1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) in DMEM/F-12 at room temperature (RT; 23°C). Time course experiments were conducted to determine the linear range of the DP-receptor-induced cyclic AMP production at 23°C. The agonist stimulus-response was linear up to 80 min under these conditions. The standard incubation protocols for test compounds were as follows: for agonists alone a 15 min incubation was used; when antagonists were tested they were incubated with the cells for a total of 60 min to allow for equilibrium to be established. The latter was based on association/dissociation studies with [^{3}H]-BWA868C binding to DP receptors, where the equilibrium for association was achieved within 60 min at 23°C (unpublished data). Ice-cold 0.1 M acetic acid (150 μl, pH 3.5) was used for the termination of cyclic AMP production and cell lysis. Finally, ice-cold 0.1 M sodium acetate (225 μl, pH 11.5 – 12.0) was added to neutralize the samples prior to analysis by radioimmunoassay (RIA).

Cyclic AMP RIA techniques have been described previously (Crider *et al*., 1998; Sharif *et al*., 1997; 1998). Briefly, experimental sample aliquot, buffer, antibody (100 μl), [^{125}I]-cyclic AMP (100 μl) additions and standard curve preparations were performed manually. Following an overnight incubation at 4°C, bound and free [^{125}I]-cyclic AMP were separated by centrifugation and manual aspiration of the supernatant. For the semi-automated RIA sample manipulation and dilution, cyclic AMP standard curve preparation, antibody and [^{125}I]-cyclic AMP additions were performed by a Biomek 1000® robot (Beckman Instruments, Fullerton, CA U.S.A.). The instrument made dilutions with RIA buffer into a 96 well filtration plate (0.45 μm surfactant-free mixed cellulose). The robot then added [^{125}I]-cyclic AMP (100 μl) to the samples followed by addition of primary antibody (100 μl). The contents were thoroughly mixed by repeated pipetting. Subsequently, an incubation of 16 – 24 h at 4°C was conducted. A secondary antibody (100 μl) was added by the robot followed by a 20 min room temperature incubation. Bound and free [^{125}I]-cyclic AMP were separated by vacuum filtration. The filters were collected using a Millipore disposable punch tip assembly and manifold. The bound [^{125}I]-cyclic AMP was measured by gamma particle analysis using a Cobra® Auto-Gamma counter (Packard Instruments, Meriden, CT, U.S.A.).

Materials

EBTr cells were purchased from the American Type Culture Collection (ATCC CCL 44, Rockville, MD, U.S.A.). Culture media and supplements were products of Life Technologies (Grand Island, NY, U.S.A.) while FBS was supplied by Hyclone (Logan, UT, U.S.A.). Ascorbic acid and IBMX were obtained from Sigma (St. Louis, MO, U.S.A.). ZK118182 ((5Z,13E) - (9R,11R,15S)0-9-chloro-15-cyclohexyl-11,15-dihydroxy -3 - oxo - 16, 17, 18, 19, 20 - pentanor-5, 13 - prostadienoic acid)) and ZK110841 ((5Z,13E)-9R,11R,15S)-9b-chlor-15-cyclohexyl -11, 15-dihydroxy -16, 17, 18, 19, 20- pentanor -5, 13-prostadienoic acid)) were generous gifts from Schering (Berlin and Bergkamen, Germany); RS-93520 ((C3′S,1R,2R,3S,6R)-2-C3′-cyclohexyl-3′hydroxyprop-1-ynyl)-3-hydroxybicyclo[4.2.0] oct-7-ylidene)butyrate)) was a generous gift from Hoffman LaRoche (Basel, Switzerland). Other prostaglandins such as AH6809 (6 - isopropoxy - 9 - oxoxanthen - 2 - carboxylic acid); SQ27986 ([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); BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl-hydantoin); enprostil (4,5,6RS)-7[(1R,2R,3R)-3-hydroxy-2-[(E)-(3R)-3-hydroxy-4-phenoxy-1-butenyl]-5-oxo-cyclopentyl]-4,5-heptadienoic acid); misoprostol, (prost-13-en-1-oic acid, 11,16-dihydroxy-16-methyl-9-oxo-,methyl ester (11a,13E); cloprostenol, (16-m-chlorophenoxy tetranor prostaglandin $F_{2\alpha}$); fluprostenol, (5Z,13E)-(9S,11R,15S)-9,11,15-trihydroxy-5,13-prostadienoic acid); PHXA85 (15R)-13,14-dihydro-17-phenyl-18,19,20-trinor-$PGF_{2\alpha}$)); U46619 (9,11-dideoxy-9a,11a-methanoepoxy prostaglandin $F_{2\alpha}$)) and BWA868C (($\pm$)-3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino) hydantoin)) were either internally synthesized or were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Early studies employed [^{125}I]-cyclic AMP RIA kits supplied by Biomedical Technologies Inc. (Stoughton, MA, U.S.A.). For the semi-automated RIA, we utilized [^{125}I]-cyclic AMP kits purchased from PerSeptive Biosystems (Cambridge, MA, U.S.A.). The 96-well filtration plates (0.45 μm surfactant-free mixed cellulose) were products of Millipore (Bedford, MA, U.S.A.).

Data analysis

Sample counts (CPM) were compared to the standard curve and cyclic AMP production was quantified by linear regression

analysis using the Microsoft Excel® software package. The EC_{50} and IC_{50} values were generated using either a non-linear iterative curve-fitting program (Michel & Whiting, 1984; Sharif *et al*., 1991) or the sigmoidal-fit function of the Origin® software packages incorporating the logistics function. The logistical equation for curve fitting was:

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

$x_0 = EC_{50}$ or IC_{50}; p = power; A_1 = minimal Y value; A_2 = maximal Y value.

When relative efficacies of various compounds were calculated they were computed as a percentage of the maximal response produced by PGD_2 since it was used a standard reference compound in the experiments. When the agonist potency of compounds apparently exceeded 100 μM, the efficacy was listed as that being produced by the highest concentration of the agonist tested, typically this being 100 μM. The pA_2 values were derived using the principles described by Arunlakshana & Schild (1959) and as recently utilized for receptor characterization (Sharif & Xu, 1996; Wiernas *et al*., 1997). When equilibrium inhibition constants (K_i) for AH6809 were calculated the equation of Cheng-Prusoff (1973) was used. A one-way ANOVA and *t*-test were used to determine significant differences in the efficacies and potencies of the DP receptor agonists.

Results

Survey of prostanoid agonist activities

Initial time-course studies showed that the PGD_2-stimulated cyclic AMP production was linear over 10–80 min at 23°C (data not shown). All subsequent experiments were conducted with agonists for 15 min at 23°C. Several different prostaglandin receptor agonists were tested for their potencies and efficacies at the DP receptor in EBTr cells (Table 1 and Figure 1). The most active DP agonists showed the following potencies (EC_{50} values): ZK118182 (16 nM), RS-93520 (23 nM), SQ27986 (33 nM), ZK110841 (33 nM), BW245C (59 nM) and PGD_2 (101 nM) ($n = 4–70$; Table 1). Compounds with activity at EP_1 (e.g. sulprostone), EP_2 (e.g. butaprost) and EP_3 (e.g. enprostil; misoprostol) receptors were weak partial agonists in the EBTr cell-line assay. $PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, and 15-cyclohexyl-$PGF_{2\alpha}$, while not very potent or efficacious, produced some stimulation of cyclic AMP, whereas the FP-receptor agents such as 17-phenyl-$PGF_{2\alpha}$, cloprostenol, fluprostenol, and PHXA85 were inactive at concentrations up to 100 μM. In addition, TP (U46619) and IP (iloprost and PGI_2) receptor agonists were weak or inactive in this adenylyl cyclase assay system (Table 1).

Studies with the DP receptor antagonists BWA868C and AH6809

Both BWA868C and AH6809 were evaluated in EBTr cells to characterize their DP antagonist characteristics. Concentration-response curves for two different agonists in the presence or absence of increasing concentrations of BWA868C are shown in Figures 2 and 3. Increasing concentrations of this antagonist produced a rightward shift in the concentration-response curves for BW245C and ZK118182 with no change in the maximal cyclic AMP production. The Schild analyses of these types of data from several experiments are shown in Figures 4 and 5. The apparent pA_2 values computed from these studies were: 8.00 ± 0.02 (slope $= 1.21 \pm 0.1$; $r = 0.986$) for BW245C and 8.14 ± 0.13 (slope $= 1.05 \pm 0.01$; $r = 0.995$) for ZK118182 ($n = 3$ for each agonist). The slopes of the Schild plots for these compounds were not statistically different from unity ($P > 0.05$).

Table 1 Potencies and efficacies of various prostaglandins in stimulating cyclc AMP formation in EBTr cells

Agonist	n	*Potency mean* EC_{50} (nM)	*s.e.m.*		*Efficacy mean* % *max*	*s.e.m.*		*Preferred receptor*
ZK118182	9	16	4	**	98	5.2	NS	DP
RS-93520	4	23	4	NS	64	8.7	***	DP
SQ27986	5	33	9	NS	101	3.0	NS	DP
ZK110841	7	33	5	*	100	2.6	NS	DP
BW245C	6	59	19	NS	121	3.2	***	DP
PGD_2	70	101	10	–	100	–	–	DP
11-deoxy-PGE_1	5	3040	480	***	20	3.0	***	EP
11-β-$PGF_{2\alpha}$	5	3060	753	***	94	3.2	***	FP
11-deoxy-16,16-dimethyl-PGE_2	5	4770	844	***	46	4.4	***	EP
15-cyclohexyl-$PGF_{2\alpha}$	3	9100	600	***	47	5.8	***	FP
PGE_2	6	>10,000	–	***	72	4.2	***	EP
$PGF_{2\alpha}$	5	>10,000	–	***	56	6.0	***	FP
PGI_2	4	>10,000	–	***	47	7.3	***	IP
13, 14-dilhydro-PGE_1	3	>10,000	–	***	24	4.3	***	EP
Enprostil	4	>10,000	–	***	19	4.7	***	EP_3
Misoprostol (SC-29333)	4	>10,000	–	***	16	3.6	***	EP_2/EP_3
17-phenyl-$PGF_{2\alpha}$	4	>10,000	–	***	7	1.2	***	FP
U46619	4	>10,000	–	***	6	2.0	***	TP
Cloprostenol	4	>10,000	–	***	4	1.4	***	FP
Sulprostone	4	>10,000	–	***	4	0.3	***	EP_1/EP_3
PHXA85	4	>10,000	–	***	3	0.9	***	FP
Iloprost	3	>10,000	–	***	3	0.9	***	IP
Butaprost	3	>10,000	–	***	2	1.0	***	EP_2
Fluprostenol	4	>10,000	–	***	2	1.0	***	FP

Potency (EC_{50}) and efficacy (E_{max} relative to PGD_2) data are shown from up to 70 independent experiments each conducted in triplicate. All prostaglandins tested were carboxylic acids except for butaprost which is a methyl ester. Compounds were tested at concentrations up to 100 μM. Efficacies for compounds with EC_{50} values greater than 10,000 nM were measured at 100 μM. NS, Not significantly different form PGD_2 ($P > 0.05$), Significantly different from PGD_2 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

In comparison with BWA868C, AH6809 demonstrated weak antagonism of the cyclic AMP formation stimulated by DP receptor agonists (Figure 6). The equilibrium dissociation constant (K_i) values for AH6809 antagonizing the PGD_2 (1 μM)- and ZK118182 (10 nM)-induced cyclic AMP production were 808 ± 193 nM ($pK_i = 6.09$; $n = 3$) and 782 ± 178 nM ($pK_i = 6.1$; $n = 4$), respectively.

Discussion

Ito *et al*. (1990) had provided the first evidence that the EBTr cells contained a DP receptor coupled to cyclic AMP production. In the present studies we have provided a detailed pharmacological characterization of this functional DP receptor using over 20 different agonist prostanoids and two different DP-receptor antagonists, thereby extending the work initiated by Ito *et al*. (1990).

The data in Table 1 emphasize the selectivity of the prostanoid receptor in EBTr cells for PGD_2 and closely related prostaglandins. Numerous compounds of the DP prostanoid class were potent and full agonists which yielded potency values from 16–101 nM for the stimulation of adenylyl cyclase activity while maintaining full efficacy. Our data agreed well with the published results for PGD_2 and ZK110841 in the EBTr cell line (Ito *et al*., 1990). Interestingly, whilst the potency ratio of BW245C and PGD_2 in the EBTr cell assay system was approximately two, the ratio observed in human platelets was close to 30 (Giles *et al*., 1989; 1991). This probably reflected differences in the efficiency and degree of receptor-effector coupling and/or differences in receptor reserves in the two systems.

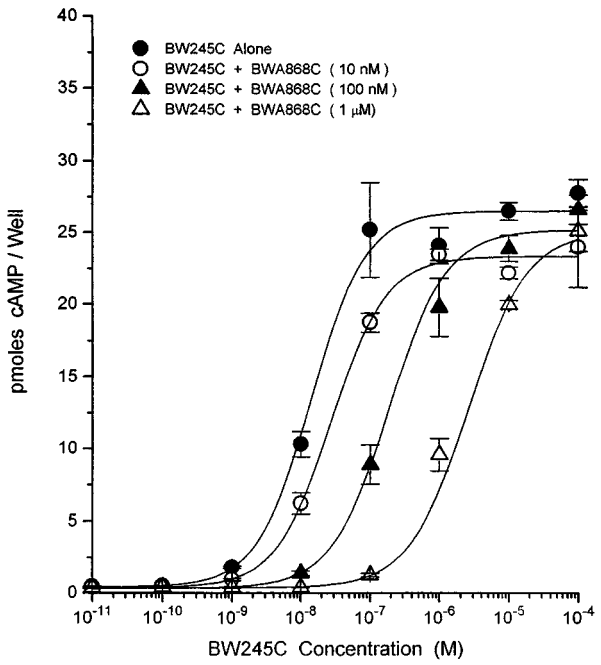

Figure 2 Concentration-response curves for BW245C stimulating cyclic AMP production in the presence of varying concentrations of the DP-receptor antagonist BWA868C in the EBTr cells. BWA868C was in contact with the cells for a total of 60 min to permit equilibrium. The cyclic AMP produced was measured by a semi-automated RIA. Data points represent means from triplicate samples from a single experiment (vertical lines show s.e.mean). Data from this and other similar experiments were transformed into Schild plots as shown in Figure 4.

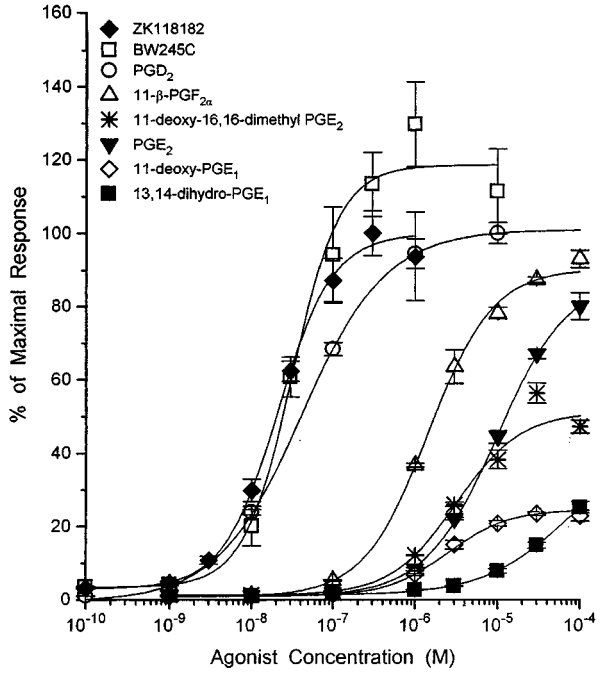

Figure 1 Representative concentration-response curves for various prostaglandins stimulating cyclic AMP production in the EBTr cells using a 15 min stimulation protocol. Cyclic AMP was measured by a semi-automated RIA. Data points represent means from triplicate samples from a single experiment (vertical lines show s.e.mean). Data from several such experiments are summarized in Table 1. The data for the different prostaglandins were normalized relative to the maximal effects of PGD_2. PGD_2 was tested as a reference compound in each agonist-response experiment.

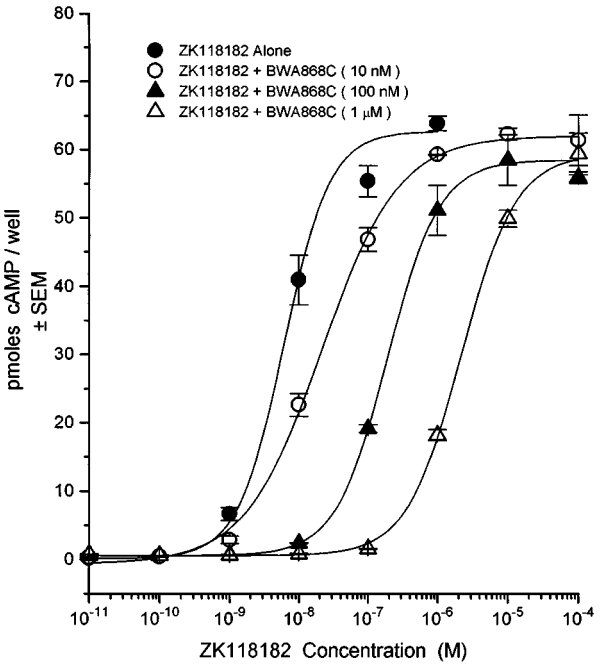

Figure 3 Concentration-response curves for ZK118182 stimulating cyclic AMP production in the presence of the DP-receptor antagonist BWA868C in the EBTr cells. BWA868C was in contact with the cells for a total of 60 min to permit equilibrium. The cyclic AMP was measured by a semi-automated RIA. Data points represent means from triplicate samples from a single experiment (vertical lines show s.e.mean). Data from this and other similar experiments were transformed into Schild plots as shown in Figure 5.

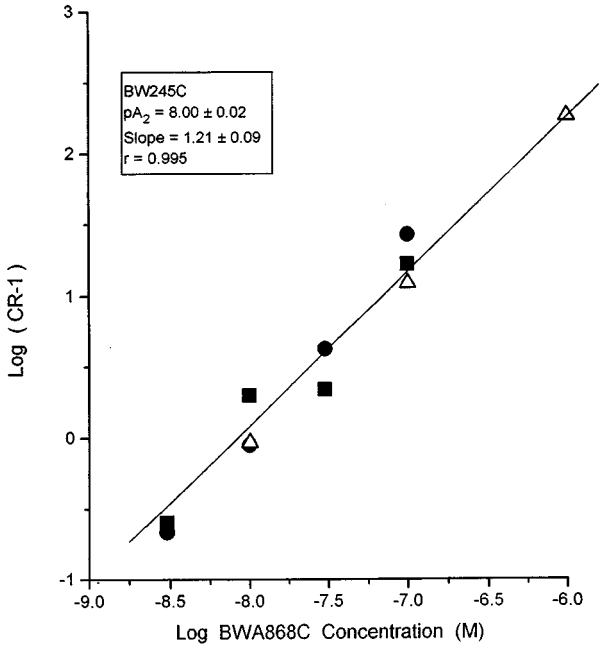

Figure 4 Schild studies with varying concentrations of the DP-receptor antagonist BWA868C versus the agonist BW245C in the EBTr cell cyclic AMP production assay. Analyses of cyclic AMP were performed by a semi-automated RIA. Data points represent means from triplicate samples from individual experiments. Linear regression line through the means of the data points is shown. Three independent experiments were performed, as indicated by the different symbols.

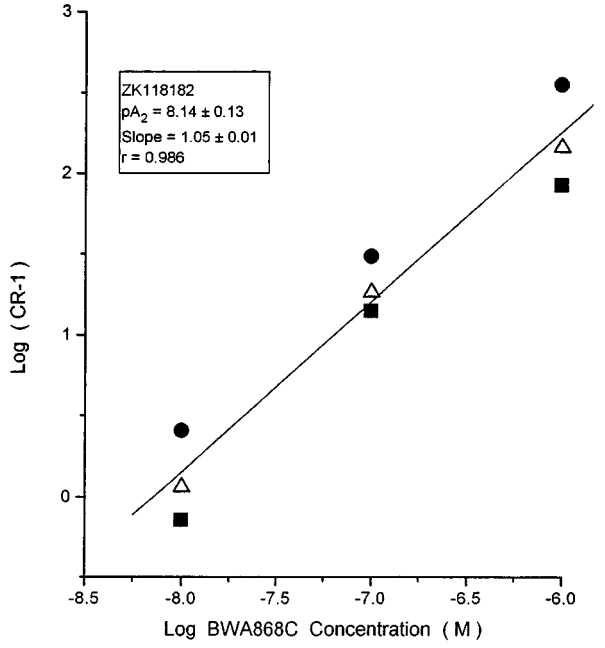

Figure 5 Schild studies with varying concentrations of the DP antagonist BWA868C versus the agonist ZK118182 in the EBTr cell cyclic AMP production assay. The cyclic AMP produced was measured by a semi-automated RIA. Data points represent means from triplicate samples from the individual experiments. Linear regression line through the means of the data points is shown. Three independent experiments were performed, as indicated by the different symbols.

RS-93520, a DP receptor agent, produced an potency value (23 nM) in the current studies for the stimulation of adenylyl cyclase that was comparable to its potency for the inhibition of human platelet aggregation (38 nM, Alvarez *et al*., 1991). This

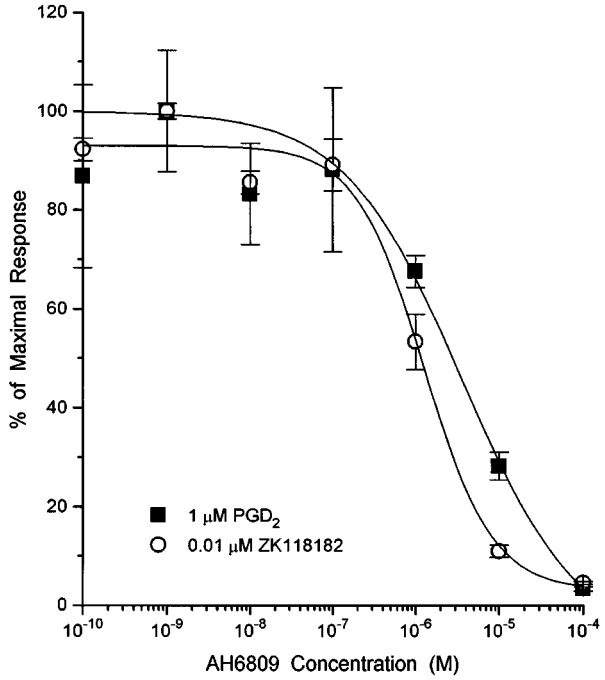

Figure 6 Representative curves for inhibition of DP-agonist-mediated cyclic AMP production by various concentrations of AH6809. The antagonist was in contact with the cells for 60 min to permit equilibrium. The cyclic AMP produced was measured by a semi-automated RIA. Data points represent means from triplicate samples from a single representative experiment (vertical lines show s.e.mean). Composite data from 3–4 similar experiments are described in the Results section.

compound was unique among the DP-receptor agonists tested in the EBTr cells because it exhibited a partial agonist profile ($E_{max} = 64 \pm 9\%$; $P < 0.001$ when compared to PGD_2). In contrast, BW245C produced about 121% of the maximal stimulation of adenylyl cyclase activity observed with PGD_2 ($P < 0.001$) in the EBTr cells. This phenomenon of increased efficacy of BW245C was also observed in HEK 293 cells expressing the recombinant human DP receptor (Boie *et al*., 1995) where it was attributed to a subpopulation of endogenous EP receptors. BW245C is known to have activity at EP_2 receptors (Giles *et al*., 1989; Matsugi *et al*., 1995). However, in our studies since PGE_1, PGE_2 and a variety of their EP-receptor-analogues were weak stimulators of the cyclase response in the EBTr cells, the apparent higher intrinsic activity of BW245C appear not to be due to stimulation of EP receptors in this cell-line. This phenomenon associated with BW245C and EBTr cell DP-receptor-effector coupling warrants further study in order to better understand the receptor reserve and intrinsic activity properties of this system.

In order to characterize the functional response of the DP receptor in the EBTr cells, prostaglandins from the other receptor classes were evaluated. Several compounds from the EP receptor class exhibited potencies approximately 100 fold less than those for the DP agonists discussed above. Therefore, a means for discriminating prostanoid agonists of different classes was provided by this assay system, with DP-receptor characteristics being the most prominent. In general, EP receptor compounds were partial agonists showing less than 100% of the maximal stimulation achieved with a DP receptor agent. As expected, the EP and IP receptor agonists produced some activity since the DP receptor shows the greatest sequence homology with receptors from this class (Boie *et al*., 1995). FP agonists showed little or no activity at the DP

prostanoid receptor, unlike PGD_2 which has considerable efficacy at FP receptors (Giles *et al*., 1991; Griffin *et al*., 1997; 1998; Sharif *et al*., 1998), and also unlike some DP agonists which are relatively potent and efficacious EP_2-receptor agonists (Crider *et al*., 1998). The rank order of potency for stimulating cyclic AMP production in the EBTr cells (ZK110841 = BW245C $\geqslant$ PGD_2 > > PGE_2 > $PGF_{2\alpha}$ > Iloprost $\cong$ U46619) is comparable to published data in HEK 293 (EBNA) cells expressing a human cloned DP receptor (Boie *et al*., 1995; Wright *et al*., 1998).

Antagonist studies

Antagonist studies with the classic DP-receptor antagonist, BWA868C, were conducted using two different DP receptor agonists, BW245C and ZK118182. BWA868C exhibited competitive antagonist characteristics with both agonists as also observed in several other cells/tissues (Giles *et al*., 1989; Trist *et al*., 1989; Senior *et al*., 1993; Lydford *et al*., 1996). In our studies, Schild plots (Figures 4 and 5) produced pA_2 values of 8.14 for ZK118182 and 8.0 for BW245C when titrated against BWA868C. This showed an agonist independent antagonism that was competitive in nature since the slopes of both lines were not significantly different from unity and maximal efficacy was observed in the presence of the antagonist under these conditions. Similar competitive antagonist effects of BWA868C have been described in human platelets, rabbit jugular vein, and human myometrium (Giles *et al*., 1989; Trist *et al*., 1989; Senior *et al*., 1993). Smooth muscle relaxation experiments with BWA868C in canine dorsal nasal vein, major palatine artery, and saphenous vein produced pK_B values of approximately 7.3, 7.6, and 7.1, respectively (Liu *et al*., 1996b). Adenylyl cyclase stimulation experiments on human platelets with BWA868C versus BW245C produced a pK_B estimate of 9.11 (Trist *et al*., 1989). However, Giles *et al*. (1989) observed pK_B estimates of 9.26 and 8.7 (BW245C versus BWA868C) in platelets and rabbit jugular vein, respectively. Additional data obtained for BWA868C in the current studies ($pK_i = 8.59$ and 8.8 versus PGD_2 and SQ27986, respectively; data not shown) and the pA_2 values mentioned above in our EBTr cell system were thus similar to those data reported for other cells and tissues mentioned above. However, a departure from a simple competitive antagonist profile was observed in platelet aggregation studies with BWA868C and BW245C that produced a Schild slope of 1.25 (Giles *et al*., 1989). The range of pK_B/pA_2 values of 7.1 – 9.3 for BWA868C noted above could have been attributed to varying experimental conditions as well as species differences. However, the possible existence of DP receptor subtypes, as has been proposed in the literature (Fernandes & Crankshaw, 1995; Rangachari *et al*., 1995), could also explain these results. Taken together, these observations warrant further studies to delineate the possible existence of multiple DP receptor subtypes.

Our studies confirm that AH6809 is a relatively weak antagonist at the DP receptor in EBTr cells just as previously reported by Ito *et al*. (1990). In our studies, AH6809 produced K_i values of approximately 808 nM ($pK_i = 6.09$) and 782 nM ($pK_i = 6.1$) when titrated against PGD_2 (1 μM) and ZK118182 (10 nM), respectively (see Figure 6 for a representative inhibition plot). AH6809 was shown to possess weak DP receptor antagonists activity in human platelet aggregation assays with a pA_2 value of 5.35 when evaluated against PGD_2 (Keery & Lumley, 1988). However, while of some pharmacological utility, AH6809 is not a selective agent since it also exhibits antagonist activity at the EP_2 receptor (Woodward *et al*., 1995; Crider *et al*., 1998). In this respect, BWA868C continues to be the most preferred DP-receptor antagonist of nanomolar affinity and potency.

In conclusion, we have employed a diverse group of prostaglandin agonists with varying potencies and receptor-selectivities to characterize the DP receptor present on EBTr cells coupled positively to adenylyl cyclase and the stimulation cyclic AMP production. The rank orders of prostaglandin potencies clearly indicated DP-receptor pharmacology. This was further borne-out by the use of two known DP-receptor antagonists, BWA868C and AH6809, which exhibited differential potencies as reported for other biological systems in the literature. The data in the current studies support the utility of the EBTr cell line, expressing an endogenous DP receptor, as a useful tool for the evaluation of various prostaglandins for their DP receptor agonist and/or antagonist properties.

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