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AL-3138 Antagonizes FP Prostanoid Receptor-mediated Inositol Phosphates Generation: Comparison with Some Purported FP Antagonists

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Abstract

The aim of this study was to pharmacologically characterize the antagonist properties of a novel prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analogue (11-deoxy-16-fluoro $PGF_{2\alpha}$; AL-3138) using a variety of second-messenger assays of prostaglandin receptor subtypes. A detailed comparison was made between AL-3138 and some purported FP receptor antagonists such as $PGF_{2\alpha}$ dimethylamine, $PGF_{2\alpha}$ dimethylamide, glibenclamide and phloretin using the FP receptor-mediated phosphoinositide turnover assay in A7r5 rat thoracic aorta smooth muscle cells and mouse Swiss 3T3 fibroblasts.

The potency and efficacy of AL-3138 as an FP receptor agonist were: $EC50 = 72{\cdot}2 \pm 17{\cdot}9$ nM ($E_{max} = 37\%$) ($n = 3$) in A7r5 cells and $EC50 = 20{\cdot}5 \pm 2{\cdot}8$ nM ($E_{max} = 33\%$) ($n = 5$) in 3T3 cells. Being a partial agonist, the antagonist potency of AL-3138 against fluprostenol in A7r5 cells was determined to be: $K_i = 296 \pm 17$ nM ($n = 3$) and $K_b = 182 \pm 44$ nM ($n = 5$) ($-\log K_b = 6{\cdot}79 \pm 0{\cdot}1$). AL-3138 exhibited very minimal or no antagonistic effects at EP_2, EP_4, DP and TP prostaglandin receptors. Both $PGF_{2\alpha}$ dimethylamide and $PGF_{2\alpha}$ dimethylamine were inactive as FP receptor antagonists, whereas phloretin and glibenclamide were very weak and had $-\log K_b$ values of $5{\cdot}28 \pm 0{\cdot}09$ ($n = 3$) and $3{\cdot}58 \pm 0{\cdot}32$ ($n = 3$), respectively. However, phloretin antagonized functional responses of EP_2 and DP prostanoid receptors, and also the V_1-vasopressin receptor. AL-3138 competed for [^{3}H]$PGF_{2\alpha}$ binding to FP receptors with a relatively high affinity ($IC50_{high} = 312 \pm 95$ nM) matching its functional antagonist potency.

In conclusion, AL-3138 is a more potent and selective FP receptor antagonist than glibenclamide, phloretin, $PGF_{2\alpha}$ dimethylamide and $PGF_{2\alpha}$ dimethylamine and is therefore a unique and novel pharmacological tool to help characterize FP receptor-mediated functions.

Prostanoids such as prostaglandins, prostacyclins and thromboxanes are potent derivatives of arachidonic acid which elicit a variety of effects including platelet aggregation, smooth muscle contraction and relaxation, lipolysis, induction of pain, luteolysis and immunoregulation (Coleman et al 1994). In the eye, certain natural and synthetic prostaglandins modulate intraocular pressure (Bito 1997), and cause iris sphincter contraction and ciliary muscle relaxation (Goh & Kishino 1994).

G protein-coupled prostanoid receptors, including DP, EP (with subtypes EP_1, EP_2 , EP_3, EP_4), FP, IP and TP receptors, mediate the diverse actions of these prostanoids (Coleman et al 1994). Prostaglandin receptors couple to a variety of effector systems and this can be summarized as follows: FP, TP and EP_1 receptors preferentially couple to $G_q/G_{q/11}$ and their activation results in the formation of inositol phosphates and diacylglycerol and subsequent mobilization of intracellular calcium (Coleman et al 1994); the DP, EP_2, EP_4 and IP receptors preferentially couple to G_s and activation of these receptors stimulates adenylyl cyclase to produce intracellular cAMP (Coleman et al 1994; Crider et al 1999, 2000). Several subtypes of EP receptors have been identified, including EP_1, EP_2, EP_3 and EP_4, which couple to various G proteins (Coleman et al 1994). Furthermore, the

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numerous splice variants of the EP_3 receptor couple to a multitude of G proteins and second messenger systems (Coleman et al 1994).

The FP prostaglandin receptor has been studied in a variety of tissues including the bovine corpus luteum (Sharif et al 1998), human uterus (Senior et al 1993), rabbit jugular vein (Chen et al 1995), various human ocular tissues (Davis & Sharif 1999), and in mouse Swiss 3T3 fibroblasts (Griffin et al 1997) and rat vascular smooth muscle cells (A7r5) (Griffin et al 1998). Potent, selective synthetic agonists at some prostaglandin receptors have been characterized in both in-vitro and in-vivo models (Coleman et al 1994). For instance fluprostenol or its enantiomer (e.g. AL-5848) (Sharif et al 1999) and cloprostenol (Coleman et al 1994; Sharif et al 1998) are potent and selective FP receptor agonists. Since most natural prostaglandins show rather limited selectivity for their preferred receptor among this receptor family, the few reported selective prostaglandin receptor agonists have been very valuable tools for discriminating discrete functional responses coupled to their respective receptors. However, conclusive identification of the particular receptors mediating prostaglandin-stimulated functional responses requires potent and selective antagonists (Kenakin 1996). Only a limited number of prostaglandin receptor antagonists are available and include BWA868C (DP antagonist; Giles et al 1989), SQ-29,548 (TP antagonist; Ogletree et al 1985), SC51089 (EP_1 antagonist), AH6809 (EP_2 and other prostaglandin receptor antagonist), and AH23848 (EP_4 antagonist) (Coleman et al 1994), the latter three being rather weak and somewhat non-selective.

The recent identification and commercial development of selective FP receptor agonists as potent and highly efficacious drugs for the treatment of elevated intraocular pressure (Bito 1997; Hellberg et al 1998) has considerably advanced our knowledge of FP receptor-coupled pharmacological actions. However, the function of the FP receptor is not fully understood, due in part to significant species differences in the tissue distribution of this receptor (Ocklind et al 1996; Davis & Sharif 1999; Sharif et al 1999). Although early studies appeared to have uncovered potential FP prostanoid receptor antagonists, for instance $PGF_{2\alpha}$ dimethylamide, $PGF_{2\alpha}$ dimethylamine (Maddox et al 1978; Stinger et al 1982), phloretin (Kitanaka et al 1993) and glibenclamide (Delaey & Van de Voorde 1995), these compounds are neither potent enough nor selective enough. We recently reported the discovery of a selective FP receptor antagonist (AL-8810) of micromolar potency (Griffin et al 1999).

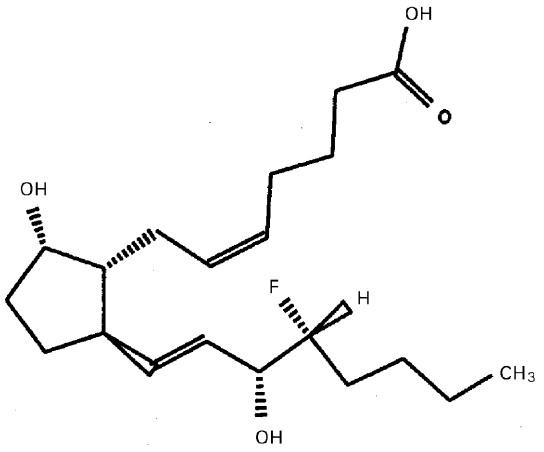

Figure 1. Chemical structure of 11-deoxy-16-fluoro $PGF_{2\alpha}$ (AL-3138).

In the present study, we describe another analogue of $PGF_{2\alpha}$ (AL-3138; Ro-22-6641; 11-deoxy-16-fluoro $PGF_{2\alpha}$) (Figure 1) which is a partial agonist of low efficacy and which also functions as an FP receptor antagonist. We compared the pharmacological properties of AL-3138 with some purported FP receptor antagonists such as phloretin, glibenclamide, $PGF_{2\alpha}$ dimethylamide and $PGF_{2\alpha}$ dimethylamine. AL-3138, being a relatively selective agent, may be a valuable FP receptor antagonist tool for investigating the specific function of the FP receptor in various biological systems.

Materials and Methods

Materials

Tissue culture and other reagents (DMEM, DMEM/F-12, Ham's F-12, glutamine, gentamicin, and trypsin/EDTA) were purchased from Life Technologies (Grand Island, NY). Foetal bovine serum (Hyclone, Logan, UT) was heat-inactivated at 56°C for 30 min and stored at −20°C. The [^{125}I]cAMP RIA kits were supplied by PerSeptive Diagnostics (Cambridge, MA). Formic acid, ammonium formate, LiCl, and phloretin were supplied by Sigma Chemical Co. (St Louis, MO). Amersham Corp. (Deerfield, IL) was the source of [^{3}H]myo-inositol. [^{3}H]$PGF_{2\alpha}$ (150–175 Ci mmol^{-1}) was purchased from New England Nuclear (Boston, MA). AG 1-X8 anion exchange resin was a product of Bio-Rad (Hercules, CA). Ecolume scintillation fluid was supplied by ICN Biomedicals (Costa Mesa, CA). Fluprostenol, $PGF_{2\alpha}$ and other standard prostaglandins were purchased from Cayman Chemical Co. (Ann Arbor, MI). Glibenclamide, tolbutamide, and tolazamide were supplied by Research Biochemicals Inc.

(Natick, MA). Arg^8-vasopressin (AVP) was purchased from Peninsula Labs. (Belmont, CA). AL-3138 (Ro-22-6641) and 11-deoxy-16-fluoro-PGE_2 (Ro-22-1322) were generous gifts from Hoffman La-Roche (Basel, Switzerland). AL-8810 (5Z, 13E)-(9S,11S,15*R*)-9,15-dihydroxy-11-fluoro-15-(2-indanyl)-16,17,18,19,20-pentanor-5,13-prostadienoic acid) was synthesized in the Medicinal Chemistry department at Alcon Research, Ltd. Glaxo-Wellcome (Stevenage, Herts, UK) generously provided BWA868C and AH23848B.

Cell culture

A7r5 rat vascular smooth muscle cells (Griffin et al 1998), Swiss mouse 3T3 fibroblasts (Griffin et al 1997), and embryonic bovine tracheal (EBTr) cells (Crider et al 1999), purchased from the American Type Culture Collection (Rockville, MD), were grown in Dulbecco's modified Eagle's medium (DMEM) containing $4{\cdot}5\,g\,L^{-1}$ glucose and $110\,mg\,L^{-1}$ sodium pyruvate, supplemented with 2 mM L-glutamine, $10\,mg\,mL^{-1}$ gentamicin sulphate, and 10% foetal bovine serum. SV-40 virus immortalized human nonpigmented ciliary epithelial (NPE) cells were grown as previously described (Crider et al 1998). Chinese hamster ovary (CHO-K1) cells (Crider et al 2000) obtained from the American Type Culture Collection were grown in a similar medium but with Ham's F-12 medium substituted for DMEM. Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air, with two changes of fresh media weekly. All cells were passaged at approximately 90% confluence by treatment with 0·05% trypsin/0·53 mM ethylenediamine tetraacetic acid (EDTA).

FP and TP receptor-mediated phosphoinositide turnover assay

Previously published procedures were used to measure [^{3}H]inositol phosphates ([^{3}H]IP) produced by FP agonist-mediated activation of phospholipase C in A7r5 cells and Swiss 3T3 cells (Griffin et al 1998, 1999). NPE cells expressing endogenous TP receptors were grown as described above. Cells grown to confluence in uncoated 24-well plastic plates were exposed for 24–30 h to 1·0–1·5 μCi [^{3}H]myo-inositol ($18{\cdot}3\,Ci\,mmol^{-1}$) in 0·5 mL DMEM without serum. Cells were then rinsed once with DMEM/F-12 containing 10 mM LiCl before incubation with agonist in the same medium for 1 h at 37°C (triplicate determinations). Antagonist effects were determined by incubating the cells with the stated concentration of the antagonist (or the solvent ethanol as a control) for 10–20 min before adding the agonist (fluprostenol, 100 nM for single concentration assays for FP; U-46619, 10 μM for single concentration assays for TP). When arginine vasopressin (AVP) was used its final concentration in the assays was 100 nM. The reactions were stopped by aspirating the medium and immediately adding 1 mL 0·1 M formic acid (at 4°C). The chromatographic separation of radiolabelled components on an AG-1-X8 column was performed as previously described (Griffin et al 1997) with sequential washes with H_2O and 50 mM ammonium formate, followed by elution of the total [^{3}H]IPs fraction with 1·2 M ammonium formate containing 0·1 M formic acid into a scintillation vial. The eluate (4 mL) was mixed with 15 mL scintillation fluid and the total [^{3}H]IPs was determined by scintillation counting on a beta counter.

DP, EP_2, and EP_4 receptor-mediated cAMP production

Detailed procedures for the measurement of prostaglandin-receptor-coupled cAMP formation by an automated radioimmunoassay (RIA) procedure have been previously described (Crider et al 1998). For this assay, various cell types (EBTr for DP and NPE for EP_2 (Crider et al 1998); CHO-K1 for EP_4 (Crider et al 2000)) were grown to confluence in uncoated 48-well plastic plates. For the agonist stimulation experiment, cells were rinsed with DMEM/F-12, and then incubated for 20 min with 0·8 mM ascorbate and 1 mM 3-isobutyl-1-methylxanthine (phosphodiesterase inhibitor) before adding the agonist (PGD_2 for DP; PGE_2 for EP_2 and EP_4 receptors). The reaction was stopped by aspirating the medium, and lysing the cells with 0·1 M acetic acid (4°C), followed by neutralization with 0·1 M sodium acetate (4°C). For the antagonist assays, AL-3138 (or other antagonist) was added to the cells 15 min before the addition of the agonists (1 μM PGD_2 for DP or 0·3–1 μM PGE_2 for EP_2 and EP_4 receptors). The cAMP assays were automated using a Biomek 1000 robot (Beckman Instruments, Fullerton, CA) to dilute samples with RIA buffer into a 96-well filtration plate (0·45 mm surfactant-free mixed cellulose), and then to add [^{125}I]cAMP and primary cAMP antibody. After thorough mixing by the robot, the samples were incubated at 4°C for 16–24 h. After robotic addition of a secondary antibody and a 20-min incubation at room temperature, bound and free [^{125}I]cAMP were separated by vacuum filtration, using a Millipore disposable punch tip assembly and manifold. The bound [^{125}I]cAMP was quantitated using a gamma counter and comparison with a standard curve of known cAMP samples included in the assay procedure.

[^{3}H]PGF$_{2\alpha}$ binding to FP receptors

[^{3}H]PGF$_{2\alpha}$ binding to the FP receptors in bovine corpus luteum membranes (BCLM) was performed as previously described (Sharif et al 1998, 1999). Briefly, total particulate bovine corpus luteum homogenates were prepared by standard homogenization (tissue disruptor setting 5 for 4 min; 15 g mL^{-1} Krebs buffer, pH 7·4) and centrifugation (30 000 g, 20 min, 4°C). The supernatants were discarded and the tissue pellets washed by two resuspension/centrifugation steps as described above. Washed BCLM total particulate homogenates (20 mg mL^{-1} in Krebs buffer; pH 7·4) were incubated with [^{3}H]PGF$_{2\alpha}$ (0·9–1·5 nM final concn) and increasing concentrations (in duplicate) of the test compound for 2 h at 23°C in a total volume of 0·5 mL as previously described (Sharif et al 1998, 1999). The nonspecific binding was defined with 10 μM unlabelled PGF$_{2\alpha}$ or cloprostenol. The assays were terminated by rapid vacuum filtration, using Whatman GF/B glass fiber filters previously soaked in 0·3% polyethyleneimine, and the receptor bound radioactivity determined by liquid scintillation spectrometry at 50% efficiency.

Data analysis

Concentration–response data were analysed by the sigmoidal fit function of a non-linear, iterative computer program (Bowen & Jerman 1995) and also of the Origin Scientific Graphics software (Microcal Software, Northampton, MA) to determine compound affinity parameters (IC50, K_i), and agonist potency (EC50) and antagonist IC50 values, respectively. The efficacy of agonists at the FP receptor was determined as a percentage of the maximum response to cloprostenol or fluprostenol, included as the FP receptor standard agonists in all experiments. Calculations of equilibrium inhibition constants were made according to Cheng & Prusoff (1973). The apparent $-\log K_b$ (antagonist dissociation constant) was calculated as: $K_b =$ (antagonist concentration)/((agonist EC50 in presence of antagonist/agonist EC50 in absence of antagonist)−1) as previously described (Arunlakshana & Schild 1989; Wiernas et al 1997; Griffin et al 1999). The ligand binding data were analysed as previously described using a non-linear, iterative computer program (Bowen & Jerman 1995; Sharif et al 1998, 1999).

Results

As shown in Figure 2, AL-3138 has partial agonist activity at the FP receptor of A7r5 cells, with mean potency (EC50) of 72·2 ± 17·9 nM (n = 3) and efficacy (E_{max}) of 37% compared with the maximum response produced by the potent, selective FP receptor agonist fluprostenol (EC50 = 4·39 ± 0·19 nM, n = 23). At the Swiss 3T3 cell FP receptor, AL-3138 was somewhat more potent (EC50 = 20·5 ± 2·8 nM, n = 5) and had similar efficacy (E_{max} = 33%) to that observed in A7r5 cells. The antagonist activity of AL-3138 at the FP receptor of A7r5 cells is shown in Figure 3; the maximum [^{3}H]IP response produced by fluprostenol was inhibited in a concentration-dependent fashion by pre-incubating the cells with AL-3138 for 10 min. The mean antagonist potency (K_i) from several such experiments was 296 ± 17 nM (n = 3) using 100 nM fluprostenol. Figure 4A shows a representative experiment with A7r5 cells showing that increasing concentrations of AL-3138 shifted the concentration–response curves of fluprostenol to the right and also decreased the maximal fluprostenol-stimulated response. The mean $-\log K_b$ determined from five experiments of this type was 6·785 ± 0·097, corresponding to a mean molar antagonist potency (K_b) value of 182 ± 43·6 nM (n = 5). AL-3138 produced very similar effects on the potency and maximum response of fluprostenol in similar experiments (Figure 4B) with Swiss 3T3 cells (Griffin et al 1997), yielding −log

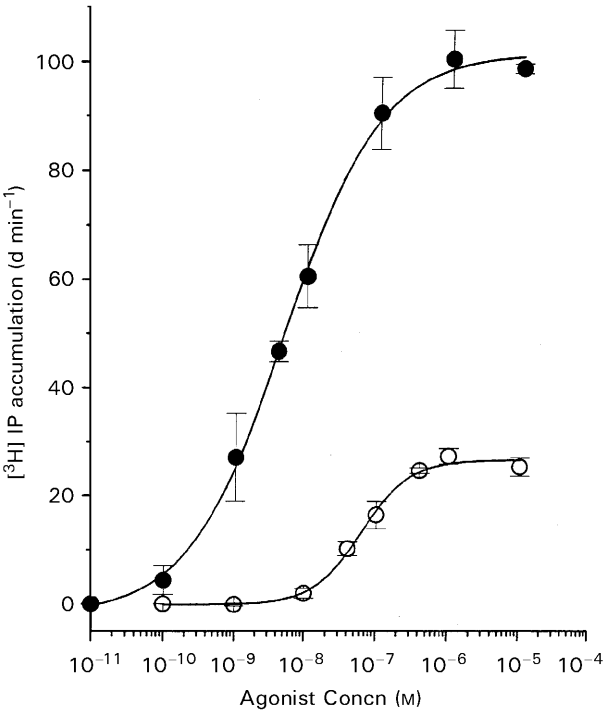

Figure 2. Agonist activity of fluprostenol and AL-3138 at the FP receptor of A7r5 cells, measured by the stimulation of [^{3}H]inositol phosphates ([^{3}H]IP) formation. A representative experiment of several experiments is shown (●, fluprostenol; ○, AL-3138). Each point is mean ± s.e.m. of triplicate determinations.

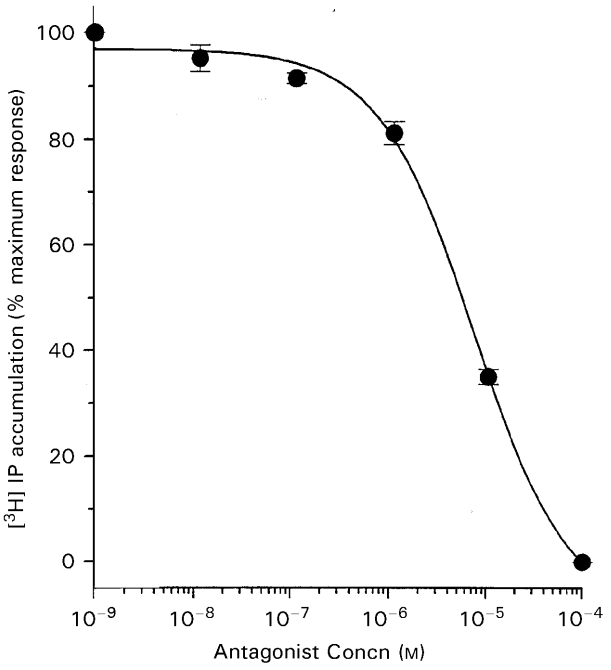

Figure 3. Concentration-dependent inhibition of fluprostenol-stimulated [^{3}H]inositol phosphates ([^{3}H]IP) formation by AL-3138 in the A7r5 cell FP receptor assay. Each point is the mean ± s.e.m. normalized response measured in three separate experiments.

$K_b = 7{\cdot}210 \pm 0{\cdot}116$ (n = 4) and $K_b = 86{\cdot}2 \pm 23{\cdot}5$ nM (n = 4). The K_b value for AL-8810, another FP antagonist was 285–426 nM (Table 1; Griffin et al 1999).

AL-3138, AL-8810 and other purported FP receptor antagonists were also evaluated for their ability to antagonize well-characterized second-messenger functional responses coupled to several other prostaglandin receptors. The results of these experiments are shown in Tables 1 and 2. These receptors were positively identified by use of highly selective antagonists, where known (e.g. TP and DP receptors) and by the ability of AH23848B to display relatively selective antagonist activity at the EP_4 receptor compared with the EP_2 receptor. For example, BWA868C, Sharif et al (2000) a known selective DP receptor antagonist, potently inhibited the DP receptor-mediated formation of cAMP in EBTr cells under conditions where AL-3138 displayed only weak inhibitory activity at this receptor (Table 2). When AL-3138 was evaluated as a functional antagonist at the TP receptor of human NPE cells, it produced only weak inhibition of [^{3}H]IP stimulated by the TP receptor agonist U-46619 (1 μM) compared with SQ-29,548, the classic potent, selective TP receptor antagonist (Table 2). AL-3138 (10 μM) produced only minimal inhibition of two adenylyl cyclase-coupled EP class receptors: the EP_4 receptor of CHO-K1 cells (< 10%) and the

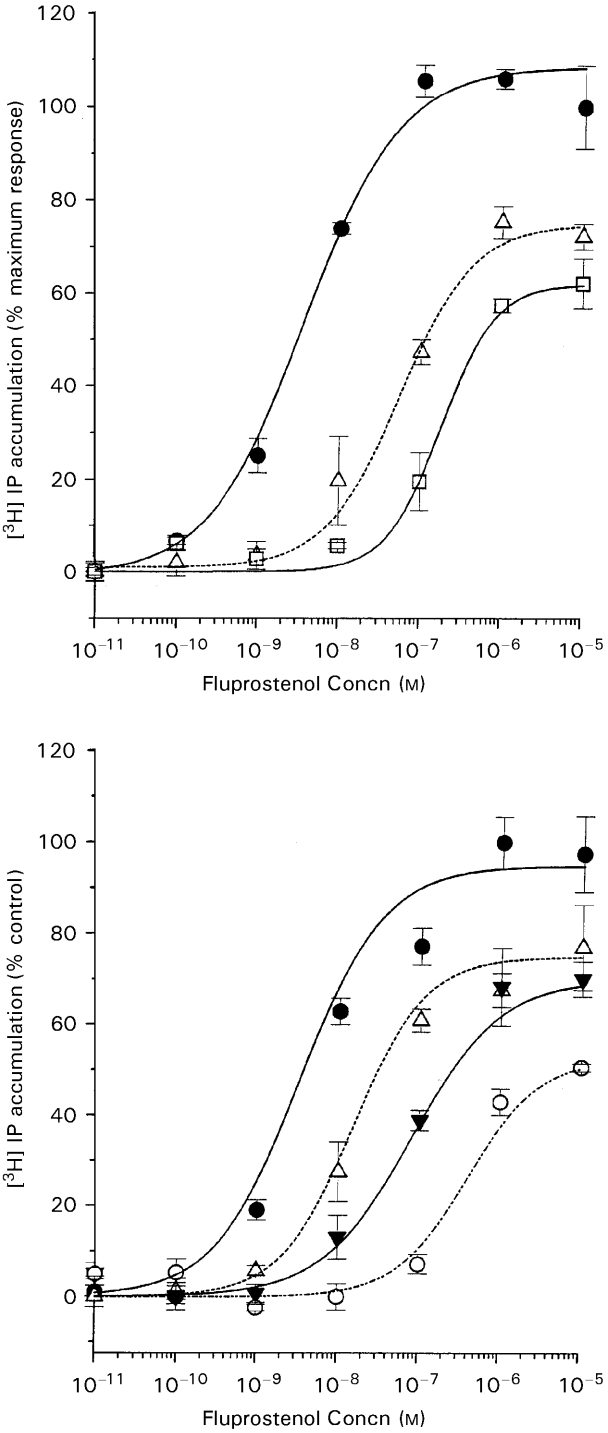

Figure 4. Effect of varying AL-3138 concentrations on the concentration–response curve of fluprostenol. A. A7r5 cells: solvent control (●) or AL-3138 (△, 1 μM; □, 10 μM). B. 3T3 cells: solvent control (●) or AL-3138 (△, 0·1 μM; ▼, 1 μM; ○, 10 μM). Each point is the mean ± s.e.m. of triplicate determinations in a single representative experiment of three performed.

EP_2 receptor of NPE cells (< 20%), under conditions where AH23848B produced significantly greater inhibition of the EP_4 receptor than the EP_2 receptor (Table 2). As further evidence for the

Table 1. Antagonist potency of AL-3138, AL-8810 and purported FP receptor antagonists at different receptors.

Antagonist	Antagonist potency (K_i or K_b [nM])					
	DP	EP_2	EP_4	FP	TP	V_1-vasopressin
AL-3138	≥100 000	≥100 000	≥100 000	86; 182–296	≥10 000	ND
AL-8810	≥30 000	≥30 000	≥30 000	285–426	≥100 000	≥100 000
Phloretin	~4250	~4229	ND	1400–5248	2119	3383

Data are the mean antagonist molar potency of compounds at the prostaglandin receptor subtypes and V_1-vasopressin receptor. ND, not determined. Glibenclamide, tolbutamide, tolazamide, $PGF_{2\alpha}$ dimethylamine and $PGF_{2\alpha}$ dimethylamide were very weak FP receptor antagonists ($K_i = 100\,000–262\,000$ nM).

Table 2. Effect of AL-3138 on the functional responses of DP, TP, EP_4 and EP_2 prostaglandin receptors.

Receptor	Antagonist concn		cAMP (% control)	Inositol phosphate (% control)
DP (EBTr cells)				
	Control (ethanol)		100	
	AL-3138	1 μM	94·6	
		10 μM	62·5	
	BWA868C	0·1 μM	22·0	
		1 μM	2·0	
TP (NPE cells)				
	Control (ethanol)			100
	AL-3138	1 μM		90·4
		10 μM		52·6
	SQ-29,548	1 μM		41·1
		10 μM		1·6
EP_4 (CHO-K1 cells)				
	Control (ethanol)		100	
	AL-3138	1 μM	104·2	
		10 μM	93·2	
	AH23848B	30 μM	23·7	
EP_2 (NPE cells)				
	Control (ethanol)		100	
	AL-3138	1 μM	102·0	
		10 μM	80·6	
	AH23848B	30 μM	74·0	

AL-3138 or other antagonists were pre-incubated with the cells for 15 min before addition of agonist. cAMP (DP, EP_4 and EP_2 receptors) or inositol phosphates (TP receptor) were measured.

unique activity of AL-3138 at the FP receptor antagonist, the corresponding PGE_2 analogue, 11-deoxy-16-fluoro PGE_2, was evaluated in the A7r5 cell FP receptor assay and the NPE cell EP_2 receptor assay. In both systems, this PGE_2 analogue was a weak agonist, with greater potency at the EP_2 receptor ($EC50 = 1\,\mu M$) than the FP receptor ($EC50 = 10{\cdot}9\,\mu M$, $E_{max} = 41\%$); however, 11-deoxy-16-fluoro PGE_2 exhibited no antagonist activity at either the FP or EP_2 receptor (data not shown).

To establish more quantitatively the significance of these results with AL-3138, we also evaluated in parallel experiments the potency and selectivity of other compounds such as phloretin and glibenclamide, which have been previously reported to antagonize FP receptor functional responses. Phloretin inhibited the fluprostenol-stimulated PI turnover response of A7r5 cells (Figure 5A) with a K_i value of $1{\cdot}40 \pm 0{\cdot}14\,\mu M$ ($n = 3$). At $30\,\mu M$, phloretin decreased both the potency and maximal response of fluprostenol (Figure 5B), with a $-\log K_b$ value of $5{\cdot}28 \pm 0{\cdot}09$ ($n = 3$). However, as shown in Figure 6, phloretin also produced demonstrable inhibition of another phospholipase C-coupled receptor (vasopressin V_1) on A7r5 cells, as well as two adenylyl cyclase-coupled prostaglandin receptors (DP and EP_2) on the mentioned cell lines (Table 1). In the presence of 0·15 mM glibenclamide, the [^{3}H]IP response of A7r5 cells to $PGF_{2\alpha}$ was characterized by a modest decrease in agonist potency and significant decrease in maximal response to $PGF_{2\alpha}$ (Figure 7); the $-\log K_b$ for glibenclamide was $3{\cdot}582 \pm 0{\cdot}315$ ($K_b = 262\,\mu M$; $n = 3$; Table 1). However, the sulphonylureas, tolbutamide and tolazamide, tested at the same concentration in the same experiments produced little or no effect on the $PGF_{2\alpha}$-stimulated response (Figure 7). $PGF_{2\alpha}$ dimethylamine was inactive as an FP agonist and $PGF_{2\alpha}$ dimethylamide displayed only very weak agonist activity ($EC50 > 100\,\mu M$) in the A7r5 cell assay (Figure 8A). Moreover, neither compound could antagonize the fluprostenol-stimulated [^{3}H]IP response of A7r5 cells even up to $100\,\mu M$ (Figure 8B).

In FP receptor-binding competition experiments, AL-3138 concentration-dependently inhibited specific [^{3}H]$PGF_{2\alpha}$ binding to BCLM membranes similar to unlabelled $PGF_{2\alpha}$ (Figure 9; Table 3). The inhibition curve for AL-3138 was shallow indicating interaction with two affinity states of the [^{3}H]$PGF_{2\alpha}$ binding sites in the BCLM membranes. Resolution of these by a two-site computer-fit model yielded the following affinity parameters: $K_{i\,high} = 312 \pm 95$ nM, $66 \pm 10\%$ of total sites; $K_{i\,low} = 19\,700 \pm 2330$ nM, $34 \pm 10\%$ of total sites.

Interestingly, AL-8810 displaced [^{3}H]$PGF_{2\alpha}$ binding in a mono-phasic manner with a Hill coefficient of unity (Figure 9; Table 3). The other purported FP receptor antagonists were very much weaker than AL-3138 and AL-8810 when competing for [^{3}H]$PGF_{2\alpha}$ binding (Figure 9; Table 3).

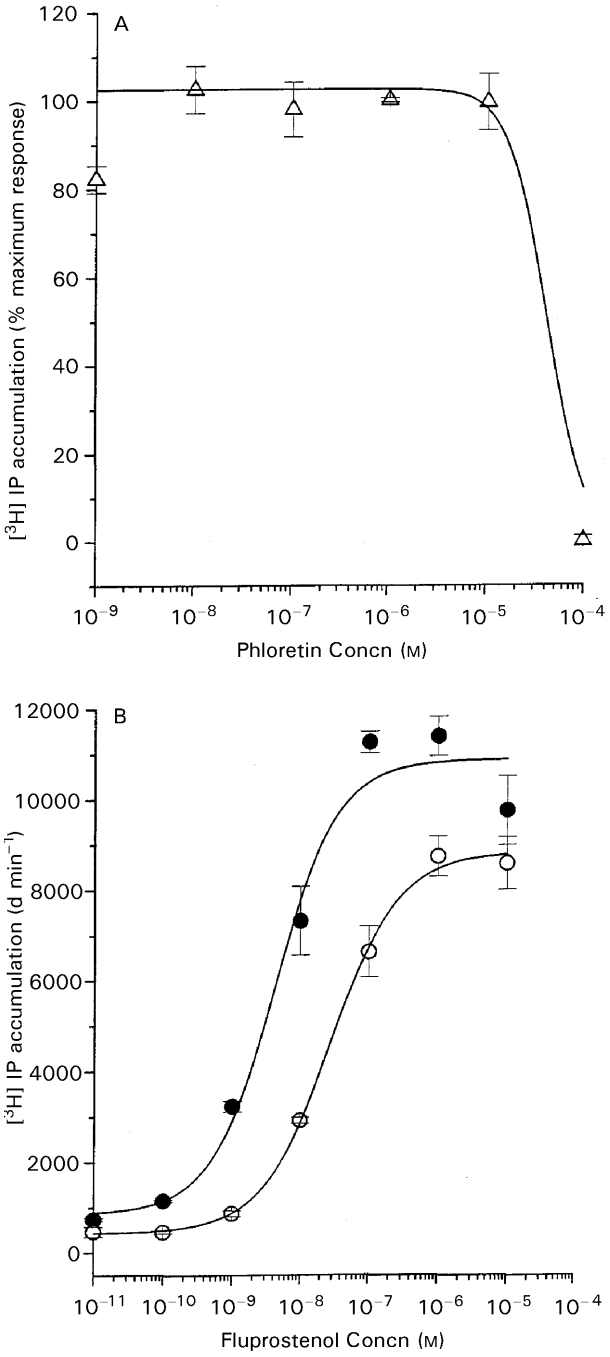

Figure 5. Effect of phloretin on fluprostenol-stimulated [^{3}H]inositol phosphates ([^{3}H]IP) accumulation by A7r5 cells. A. Concentration of phloretin was varied against a fixed concentration (100 nM) of fluprostenol. B. Fluprostenol concentration was varied in the presence of ethanol (control; ●) or 30 μM phloretin (○). Each point is the mean ± s.e.m. of triplicate determinations. A representative experiment of three performed of each type is shown.

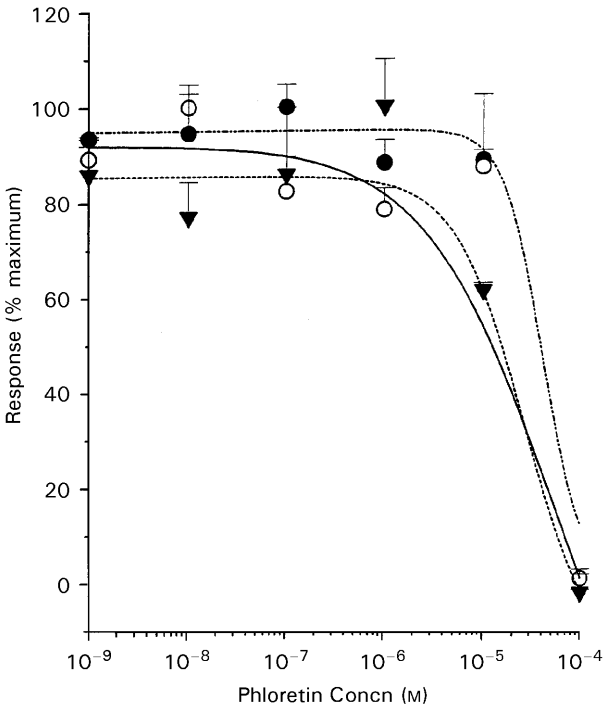

Figure 6. Effects of phloretin on functional second-messenger responses mediated by prostanoid and non-prostanoid receptors (○, PGE_2-stimulated cAMP formation in NPE cells; ▼, PGD_2-stimulated cAMP formation in embryonic bovine tracheal cells; ●, AVP-stimulated [^{3}H]inositol phosphates ([^{3}H]IP) accumulation by A7r5 cells). Each point is the mean ± s.e.m. normalized response of triplicate determinations in a single experiment. A representative experiment of three performed with each system is shown.

Discussion

In this study, the functional activity of AL-3138 at the endogenous FP receptors of A7r5 rat vascular smooth muscle cells and Swiss 3T3 albino mouse fibroblasts has been characterized in detail. In each cell line, AL-3138 was both a potent partial agonist of relatively low efficacy and a functional antagonist at the FP receptor when tested in the antagonist protocol. AL-3138 evaluated at a fixed concentration in the antagonist protocol decreased the potency of fluprostenol, a potent, selective FP receptor agonist, and also decreased the maximal agonist-induced response. These results suggest that the antagonistic action of AL-3138 at the FP receptor has apparent non-competitive characteristics. Based on agonist potency (EC50 values of 20·5 nM vs 72·2 nM) and antagonist K_b values (86·2 nM vs 182 nM), AL-3138 appeared to have a greater potency at the mouse FP receptor (3T3 fibroblasts) than at the rat FP receptor (A7r5 cells). However, the significance of these observations is unclear and requires further study.

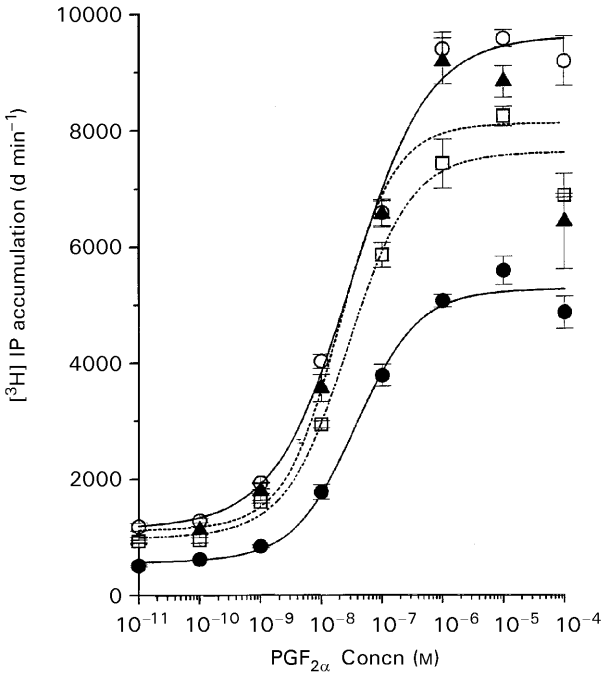

Figure 7. Effect of glibenclamide and other sulphonylurea drugs on fluprostenol-stimulated [^{3}H]inositol phosphates ([^{3}H]IP) formation in A7r5 cells. Concentration–response curves to the agonist were generated in the presence of glibenclamide (●), tolazamide (▲), tolbutamide (□) and DMSO (control; ○). Each point is the mean ± s.e.m. normalized response of triplicate determinations in a single experiment. A representative experiment of three performed with each compound is shown.

Since at present there is no FP receptor antagonist with well established pharmacological properties, apart from our recently described FP antagonist, AL-8810 (Griffin et al 1999), we studied several other compounds purported to be FP receptor antagonists in FP ligand binding and various prostanoid receptor functional assays. Among this group of FP receptor antagonists, the dimethylamine and dimethylamide analogues of $PGF_{2\alpha}$ did not exhibit any antagonist activity at the FP receptor and were not potent inhibitors of [^{3}H]$PGF_{2\alpha}$ binding in our experiments. The early reports of antagonist activity of both compounds made use of isolated tissue preparations and very high concentrations of these lipophilic compounds (Maddox et al 1978; Stinger et al 1982), which may have damaged the tissue or interfered with tissue penetration of the agonist. To our knowledge, there have been no confirmed reports of antagonistic effects of $PGF_{2\alpha}$ dimethylamine and $PGF_{2\alpha}$ dimethylamide at the FP receptor.

The results of this study showed glibenclamide to be significantly less potent than AL-3138 as an FP receptor antagonist and as a competitor of [^{3}H]$PGF_{2\alpha}$ binding to FP receptors. Glibenclamide has been previously reported to antagonize the

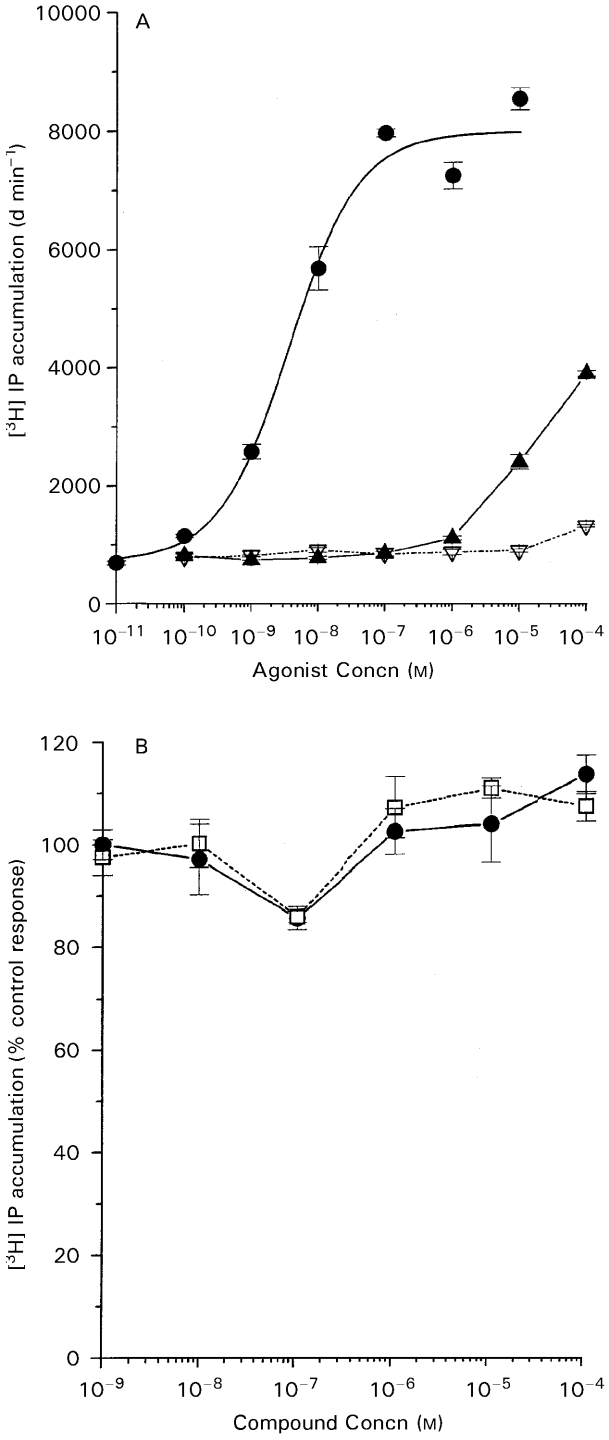

Figure 8. Effects of $PGF_{2\alpha}$ dimethylamide and $PGF_{2\alpha}$ dimethylamine evaluated as agonists and antagonists at the FP receptor of A7r5 cells. A. Agonist protocol. Concentration–response curves to fluprostenol (●), $PGF_{2\alpha}$ dimethylamide (▲) and $PGF_{2\alpha}$ dimethylamine (▽) were generated using PI assays. B. Antagonist protocol. The effects of $PGF_{2\alpha}$ dimethylamide (●) and $PGF_{2\alpha}$ dimethylamine (□) on fluprostenol-mediated phosphoinositide turnover were studied. In both plots, each point is the mean ± s.e.m. response of triplicate determinations in one representative experiment of three performed.

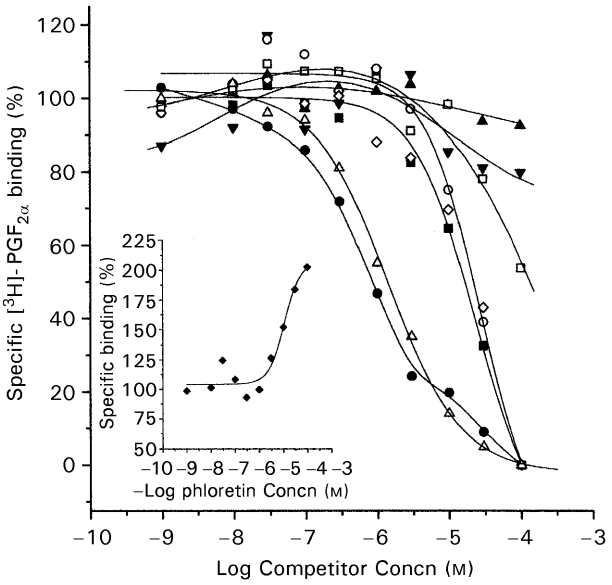

Figure 9. Competition for specific [^{3}H]$PGF_{2\alpha}$ binding by various compounds to FP receptors in bovine corpus luteum membranes. The relative affinity of $PGF_{2\alpha}$, AL-3138, AL-8810 and putative FP receptor antagonists for the FP receptor was determined using a range of concentrations of the competing compounds (●, AL-3138; △, $PGF_{2\alpha}$; ■, AL-8810; ◇, $PGF_{2\alpha}$ dimethylamide; ○, glibenclamide; □, $PGF_{2\alpha}$ dimethylamine; ▼, tolazamide; ▲, tolbutamide). Data shown are from a representative experiment, but composite data from numerous such experiments are shown in Table 3. The inset shows the paradoxal stimulation of [^{3}H]$PGF_{2\alpha}$ binding by phloretin.

Table 3. Inhibition of [^{3}H]$PGF_{2\alpha}$ binding to FP prostaglandin recepors in bovine corpus luteum membranes.

Compound	Inhibition of [^{3}H]$PGF_{2\alpha}$ binding (K_i, nM)		Hill coefficient (n_H)
$PGF_{2\alpha}$	130 ± 6	(n = 130)	0·90 ± 0·01
AL-3138	2600 ± 1300	(n = 3)	0·55 ± 0·11[a]
AL-8810	13 000 ± 590	(n = 3)	1·01 ± 0·18
$PGF_{2\alpha}$ dimethyl-amide	14 000 ± 1800	(n = 3)	1·09 ± 0·16
Glibenclamide	18 000 ± 530	(n = 5)	–
Tolazamide	> 100 000	(n = 2)	–
Tolbutamide	> 100 000	(n = 2)	
$PGF_{2\alpha}$ dimethyl-amine	110 000 ± 25 000	(n = 3)	0·95 ± 0·1
Phloretin	Stimulated binding (n = 3)		–

Data are mean ± s.e.m. [a]For AL-3138 the Hill coefficient was significantly different from unity and further analyses of data by two-site fit analysis yielded the following data: $IC50_{high}$ = 312 ± 95 nM; $IC50_{low}$ = 19 700 ± 2330 nM. Phloretin consistently increased the binding of [^{3}H]$PGF_{2\alpha}$. The potency of phloretin at stimulating [^{3}H]$PGF_{2\alpha}$ binding to the FP receptor was 6·5 ± 2·4 μM (n = 3).

contractile response of isolated rat aorta preparations to $PGF_{2\alpha}$, PGE_2 and U-46619, all mediated presumably by the FP receptor (Delaey & Van de Voorde 1995). In those studies, contractions induced by prostaglandins were more sensitive to inhibition by glibenclamide than tolbutamide but contractile responses to other agonists were not affected by either sulphonylurea. It was proposed that glibenclamide exerted its antagonistic actions in these rat aorta preparations at the level of G proteins (Delaey & Van de Voorde 1995). The relative potency of glibenclamide and tolbutamide observed by Delaey & Van de Voorde (1995), which was confirmed in our studies on FP receptors, is consistent with the relative potency of these compounds as ATP-regulated K^+ channel blockers (Loffler-Salz & Quast 1998). However, the absolute potency of glibenclamide, in particular, as a prostaglandin receptor antagonist is rather low, compared with its potent pharmacological activity at ATP-sensitive K^+ channels. Glibenclamide has been shown to bind to the sulphonylurea receptor protein subunit of the K^+-ATP channel protein with a K_i value of 100–200 nM, whereas tolbutamide typically exhibits at least 100-fold lower potency than glibenclamide at this channel (Loffler-Salz & Quast 1998). However, glibenclamide was recently reported to produce not only specific effects but also non-specific effects on K^+ fluxes (Jaburek et al 1998). Based on those findings, as well as the reported antagonistic effects of glibenclamide at certain other prostaglandin receptors (Zhang et al 1991; Deachapunya & O'Grady 1998), we conclude that non-specific effects requiring excessive concentrations of glibenclamide are responsible for its weak antagonistic activity at the FP receptor. As an FP receptor antagonist, AL-3138 is clearly more potent and selective, and has a higher FP receptor binding affinity, than glibenclamide.

Phloretin is a naturally-occurring flavonoid with reported biological activity (inhibitor of glucose and urea transport (Bissonnette et al 1996; Couriaud et al 1996); inhibitor of protein kinase C (Li et al 1998); a weak oestrogen mimetic (Breinhlot & Larsen 1998)). Phloretin was previously shown to inhibit $PGF_{2\alpha}$-induced phospholipase C activity and intracellular calcium mobilization in cultured rat astrocytes (Kitanaka et al 1993) without affecting responses to norepinephrine, carbachol, endothelin-1, glutamate or ATP (Kitanaka et al 1993). However, in our study, phloretin inhibited phosphoinositide turnover responses coupled to both the FP receptor and the vasopressin V_1 receptor in A7r5 cells, as well as other prostaglandin-receptor mediated responses, for example, activation of adenylyl cyclase by the DP and EP_2 receptor in other cell types. Hence, it was neither a potent nor selective antagonist. Furthermore, phloretin enhanced the binding [^{3}H]$PG_{2\alpha}$ to corpus luteal FP receptors instead of inhibiting it like other antago-

nists, suggesting a complex interaction between phloretin and the FP receptor binding site.

In conclusion, we have characterized the FP receptor antagonist activity of AL-3138, a newly discovered FP receptor partial agonist, at the endogenous FP receptors of A7r5 rat vascular smooth muscle cells and Swiss 3T3 albino mouse fibroblasts. This compound is more potent and more selective as a functional FP receptor antagonist than purported FP receptor antagonists, such as, phloretin and glibenclamide, which were directly compared with AL-3138 in our study. The functional antagonist potency of AL-3138 and its affinity for the high-affinity [^{3}H]$PG_{2\alpha}$ binding site matched well indicating that AL-3138 interacts at the FP receptor in a direct manner. Therefore, due its high affinity, potency and relative FP receptor selectivity AL-3138 may prove be a useful pharmacological tool to study the functions of the FP prostaglandin receptor.

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