J. Pharm. Pharmacol. 1999, 51: 685–694
Received November 27, 1998
Accepted January 13, 1999

[^{3}H]AL-5848 ([^{3}H]9β-(+)-Fluprostenol). Carboxylic Acid of Travoprost (AL-6221), a Novel FP Prostaglandin o study the Pharmacology and Autoradiographic Localization of the FP Receptor

N. A. SHARIF, T. L. DAVIS AND G. W. WILLIAMS

Molecular Pharmacology Unit, Alcon Laboratories, Inc., 6201 South Freeway, Forth Worth, TX 76134-2099, USA

Abstract

AL-5848 (5*Z*,13*E*)-(9 *S*,11*R*,15*S*)-9,11,15-trihydroxy-5,13-prostadienoic acid) is the carboxylic acid of travoprost (AL-6221), a single (+)-isomer of (±)-fluprostenol, an FP-class prostaglandin agonist which lowers intraocular pressure. We have prepared a radioligand from this selective prostaglandin and demonstrated its utility for studying the pharmacology and autoradiographic location of the FP-receptor. Specific [^{3}H]AL-5848 binding (84% of total) was linearly related to bovine corpus luteum tissue concentration and reached equilibrium within 275 min at 23°C. Scatchard analysis of saturation isotherms indicated interaction of [^{3}H]AL-5848 with a single class of high-affinity (dissociation constant, K_d, = 33·8 ± 2·9 nM, n = 4) and saturable (B_{max} = 37·3 ± 3·0 pmol (g wet weight tissue)$^{-1}$) FP receptor-binding sites in bovine corpus luteum. Specific [^{3}H]AL-5848 binding was potently inhibited by the FP-receptor ligands 16-phenoxy$PGF_{2\alpha}$ (inhibition constant K_i = 17·3 nM); cloprostenol (K_i = 56·8 nM); 17-phenyl $PGF_{2\alpha}$ (K_i = 87·0 nM); AL-5848 (K_i = 52·1 nM); $PGF_{2\alpha}$ (K_i = 195 nM); PHXA85 (K_i = 223 nM); (n = 3–11) but very weakly by PGD_2, ZK118182, BW245C, PGE_2, PGI_2 and U-46619. The pharmacology of specific [^{3}H]AL-5848 binding correlated well with the pharmacology of [^{3}H]$PGF_{2\alpha}$ binding in the bovine corpus luteum preparation (r = 0·98, n = 14, $P < 0·0001$) and also with functional responses in Swiss 3T3 and rat vascular smooth muscle cells (A7r5) (r = 0·96) expressing FP receptors. Autoradiographic studies revealed high levels of specific FP-receptor binding with [^{3}H]AL-5848 on granulosa cells in the bovine corpus luteum sections, and on longitudinal ciliary muscle, the ciliary process, the iris sphincter and the retina in eye sections from man.

These studies show [^{3}H]AL-5848 to be a high-affinity agonist radioligand capable of selectively labelling the FP prostaglandin receptor.

Prostanoids such as prostaglandins, prostacyclins and thromboxanes are potent autocoids, derived from arachidonic acid, which have many physiological and pathological effects in mammalian cells and tissues (Coleman et al 1994). Several different types of naturally occurring prostaglandin (for example the A, B, D, E and F series, etc.) are known. Depending on the number of double bonds in the α (top) and ω (bottom) chains, the prostaglandins are further classified with subscripts such as PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$, etc. (Coleman et al 1994). Although these classes of prostaglandin interact preferentially with the designated major classes of receptor (e.g. DP, EP, FP) and subclasses (e.g. EP_1, EP_2, etc.), the subscripts associated with the prostaglandin do not necessarily correspond with the subclasses of the receptor(s) with which they interact. Furthermore, it is well known that these endogenous prostaglandins are somewhat non-specific in terms of binding with various classes of prostaglandin receptors (Coleman et al 1994). However, various synthetic prostaglandins do have significant receptor selectivity (see below). Some key actions of prostanoids include lipolysis, platelet aggregation (Andersen & Ramwell 1974), smooth-muscle contraction and relaxation, induction of pain (Coleman et al 1994), intraocular

Correspondence: N. A. Sharif, Molecular Pharmacology Unit, Alcon Laboratories, Inc. (R2-19) 6201 South Freeway, Fort Worth, TX 76134-2099, USA.

pressure regulation (Wang et al 1990), iris sphincter contraction (Goh & Kishino 1994), ciliary muscle relaxation (Goh & Kishino 1994), luteolysis and immunoregulation (see Coleman et al 1994 for review). Cell-surface receptors which have different selectivity for natural prostaglandins such as PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TXA_2 (thromboxane A_2) (Coleman et al 1994; Narumiya 1994) mediate these different physiological and pharmacological effects of endogenous and synthetic prostanoids.

The current prostaglandin receptor nomenclature and classification, as defined by pharmacological and molecular cloning techniques: DP, EP (with further subtypes EP_1, EP_2, EP_3, EP_4), FP, IP and TP receptors, reflect their preferred series of prostaglandins (Coleman et al 1994). Thus, DP receptors interact preferentially with D-series prostaglandins (e.g. PGD_2) and FP receptors interact preferentially with F-series prostaglandins (e.g. $PGF_{2\alpha}$). Splice variants of the EP_3 receptor have also been discovered recently; these are EP_{3A}, EP_{3B}, EP_{3c}, and EP_{3D} (Coleman et al 1994; Narumiya 1994). Prostaglandin receptors couple with a variety of effector systems. The FP, TP and EP_1 receptors couple preferentially to $G_q/G_{q/11}$ and their activation results in the formation of inositol trisphosphate and diacylglycerol and mobilization of intracellular Ca^{2+} (Coleman et al 1994; Abramovitz et al 1995). The DP, EP_2, EP_4 and IP receptors couple preferentially to G_s and activation of these receptors stimulates adenylyl cyclase to produce intracellular cAMP (Narumiya 1994; Abramovitz et al 1995). Several subtypes of EP receptor have been identified, including EP_1, EP_2, EP_3 and EP_4, which couple to various G proteins (see Coleman et al 1994 for review). Furthermore, the numerous splice variants of the EP_3 receptor couple to a multitude of G proteins and second-messenger systems (Coleman et al 1994; Narumiya 1994; Abramovitz et al 1995).

The FP prostaglandin receptor has been studied in a variety of tissues including bovine corpus luteum (Niswender & Nett 1988; Chegini et al 1991; Sharif et al 1998), the uterus from man (Senior et al 1993; Fernandes & Crankshaw 1995), the rabbit jugular vein (Chen et al 1995), iris sphincter muscle (Goh & Kishino 1994; Abdel-Latif 1995) and a variety of cells such as Swiss 3T3 fibroblasts (Griffin et al 1997) and rat vascular smooth muscle cells (A7r5) (Griffin et al 1998). In the binding and autoradiographic studies conducted thus far on FP receptors $[^3H]PGF_{2\alpha}$ has been employed as the only commercially available radioligand when the constitutive (Hammarstrom et al 1976; Chegini et al 1991) or cloned (Sakamoto et al 1994) FP receptor has been characterized. $PGF_{2\alpha}$ is rather a promiscuous prostaglandin (Coleman et al 1994)—in bovine ciliary body membranes $[^3H]PGF_{2\alpha}$-labelled sites were more potently displaced by PGE_2 than by $PGF_{2\alpha}$ (Csukas et al 1993) and in bovine corpus luteum homogenates biphasic interactions of $[^3H]PGF_{2\alpha}$ were detected (Sharif et al 1998), making the interpretation of the binding sites somewhat complicated. In addition, $[^3H]PGF_{2\alpha}$, a natural prostaglandin, is subject to relatively rapid degradation, especially when broken-cell preparations are used in ligand-binding studies. Hence, $[^3H]PGF_{2\alpha}$ is not an ideal radioligand for labelling FP-receptor binding sites. To overcome these problems we decided to radiolabel the (+) isomer (AL-5848) of (±)-fluprostenol, a potent and selective FP-receptor agonist, the isopropyl ester of which (AL-6221) is a potent intraocular pressure-lowering prostaglandin (Hellberg et al 1998; Sallee et al 1998). We report herein the FP-receptor binding characteristics of $[^3H]$AL-5848 and its use in quantitative autoradiographic studies to visualize the FP-receptor binding sites in thin sections of bovine corpus luteum and eyes from man.

Materials and Methods

Materials

$[^3H]$AL-5848 (19·5 Ci mmol^{-1}; 2 mCi mL^{-1}) was custom-radiolabelled by Dupont-NEN (Boston, MA); $[^3H]PGF_{2\alpha}$ (150–175 Ci mmol^{-1}) was purchased from Dupont-NEN (Boston, MA); and $[^3H]$Microscale radioactivity standards and radiation-sensitive Hyperfilm for autoradiographic studies were purchased from Amersham Life Science (Arlington Heights, IL). Kodak D19 and Kodafix were purchased from a local photography shop. Human donor eyes were obtained from local eye banks. Frozen bovine corpus lutea from numerous cows of unknown menstrual and hormonal status were obtained from Pel-Freez (Rogers, AR). The prostaglandins ZK118182 and S-1033 were generous gifts from Schering (Berlin and Bergkamen, Germany) and Shionogi (Osaka, Japan), respectively, and UF-021 was synthesized by our colleagues in the Medicinal Chemistry department. The other prostaglandins used in our studies were purchased from Cayman (Ann Arbor, MI). All other standard reagents, chemicals and buffers were purchased from Sigma (St Louis, MO). Phosphorimager (Cyclone) was purchased from Packard Instruments (Meridin, CT). Agfa Arcus II digital scanner was purchased from Agfa-Gevaert (Boston,

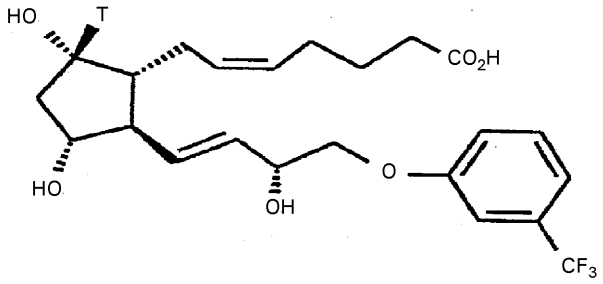

Figure 1. The structure of [^{3}H]AL-5848. T depicts the position of the tritium atom.

MA). Tissue-Tek material was from Miles (Elkhart, IN).

Preparation of [^{3}H]AL-5848

A full description of the synthesis and radiolabelling of [^{3}H]AL-5848 will be given elsewhere. The structure shown in Figure 1 depicts where the tritium atom was incorporated in the molecular structure of AL-5848.

[^{3}H]PGF$_{2\alpha}$ binding assays

Details of tissue preparation and the [^{3}H]PGF$_{2\alpha}$ binding assay have recently been described (Sharif et al 1998). In brief, 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* for 20 min at 4°C) procedures. Supernatant was discarded and the tissue pellets washed by two re-suspension-centrifugation steps as above. Washed bovine corpus luteum total particulate homogenates (20 mg mL^{-1} in Krebs buffer; pH 7·4; see above) were incubated with [^{3}H]PGF$_{2\alpha}$ (0·9–1·5 nM final) 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). The non-specific binding was defined with 10 μM unlabelled PGF$_{2\alpha}$ or cloprostenol. Standard time-courses of association of [^{3}H]PGF$_{2\alpha}$ binding to bovine corpus luteum homogenates were also conducted at 37°C for comparison with association of [^{3}H]AL-5848 (see below). The assays were terminated by rapid vacuum filtration through Whatman GF/B glass fibre filters previously soaked in 0·3% polyethyleneimine, and the receptor-bound radioactivity was determined by liquid scintillation spectrometry at 50% efficiency.

[^{3}H]AL-5848 binding assays

The tissue preparation and ligand binding assays for [^{3}H]AL-5848 were essentially identical with those described above for [^{3}H]PGF$_{2\alpha}$ binding to bovine corpus luteum homogenates except that after the time-course determinations the assays were conducted for 3·5 h at 23°C. For time-course, tissue linearity and competition experiments, a low concentration (5 nM final) of [^{3}H]AL-5848 was used with non-specific binding being defined with 10–100 μM unlabelled PGF$_{2\alpha}$ or (±)-fluprostenol. For saturation experiments the concentrations of [^{3}H]AL-5848 were varied from 1 to 110 nM. The assays were terminated by rapid vacuum filtration through Whatman GF/B glass fibre filters previously soaked in 0·3% polyethyleneimine, and the receptor-bound radioactivity was determined by liquid scintillation spectrometry.

DP-, EP$_3$-, IP- and TP-class binding assays

[^{3}H]PGD$_2$ was used to label DP receptors on platelets from man as previously described (Cooper & Ahern 1979). EP$_3$ receptors were studied in bovine corpus luteum membranes by use of [^{3}H]PGE$_2$ (Sharif et al 1998), and IP and TP receptors were labelled on platelets from man as described elsewhere by use of [^{3}H]iloprost (Armstrong et al 1989) and [^{3}H]SQ29548 (Ogletree & Allen 1992), respectively. All these ligand binding assays were performed at 23°C.

Receptor autoradiographic studies

Eyes from human cadavers, 24–79 years; mean age 49 ± 9 years (mean ± s.e.m.; n = 6) were obtained, on ice, from the Central Florida Eye Bank and some local eye banks. They were frozen on to microtome chucks in Tissue-Tek O.C.T embedding material within 24 h of death. None of the donors had any documented ocular diseases. Freshly isolated frozen bovine corpus lutea were obtained from Pel-Freez and also frozen on chucks as above. Tissue sections (20 μm) were cut from eyes at −17°C on a freezing microtome and collected on gelatinized glass microscope slides (Sharif & Hughes 1989; Blue et al 1995; Sharif 1996). The sections were pre-incubated in Tris-HCl (pH 7·4, 50 nM, 550 mL) containing NaCl (100 nM), $CaCl_2$ (3 nM) and 5% bovine serum albumin (BSA; fraction V) for 1 h at 23°C (Matsuo & Cynader 1992; Davies & Shariff 1999). The slides were then covered with [^{3}H]AL-5848 solution (40 nM, 1 mL) prepared in the above buffer with 1% BSA containing either 100 μM unlabelled PGF$_{2\alpha}$ or cloprostenol to define non-specific binding, or none of the unlabelled compounds to define the total binding. After incubation at 23°C for 3·5 h the solutions were poured off the slides and the slides were

rinsed in ice-cold buffer (550 mL, see above, containing 1% instead of 5% BSA) on a rotary mixer for 10 min. The slides were then dried in a stream of cool air and placed in a vacuum desiccator overnight. Autoradiograms from the sections and [^{3}H]Microscale radiation standards were generated over 19 days and quantified by image analysis using the phosphor-imager, Cyclone, and the associated Optiquant software package, using the principles previously described (Sharif 1996; Upham & Englert 1998; Davies & Shariff 1999). Some of the higher-resolution images were generated on tritium-sensitive Hyperfilm (Sharif & Hughes 1989) after 27 weeks exposure and scanned via an Agfa Arcus II digital scanner.

Data analyses

The original data (disintegrations min^{-1}) from the different ligand-binding experiments were analyzed by use of non-linear, iterative curve-fitting computer programs incorporating logistic functions (Sharif et al 1991; Bowen & Jerman 1995). Additional analyses were performed by use of another suite of computer programs (McPherson 1983). The inhibition constants (K_i) were calculated from IC50 values (amounts resulting in 50% inhibition) as described elsewhere (Cheng & Prusoff 1973). Autoradiographs were quantified by use of the principles of image analysis (Sharif 1996) via the Optiquant software package (Upham & Englert 1998; Davies & Shariff 1999).

Results

AL-5848 was found be significantly more FP-receptor-selective than $PGF_{2\alpha}$ when tested in a battery of PG receptor-binding assays (Table 1). AL-5848 was also a more potent agonist than $PGF_{2\alpha}$ in stimulating phosphoinositide turnover in Swiss 3T3 fibroblasts. Thus AL-5848 EC50 (concentration with 50% of maximum effect) = $4 \pm 0{\cdot}5$ nM (n = 5) and $PGF_{2\alpha}$ EC50 = $24 \pm 4{\cdot}6$ nM (n = 8) (data not shown).

To label the FP receptor binding sites, [^{3}H]AL-5848 ($19{\cdot}5$ Ci $mmol^{-1}$; 2 mCi mL^{-1}) was generated by tritium exchange of a suitable precursor and shown to be > 95% pure by high-performance liquid chromatography and thin-layer chromatography. Specific [^{3}H]AL-5848 (5 nM) binding to washed total particulate homogenates of bovine corpus luteum (a tissue enriched in FP receptors), was linear over the range 10–60 mg mL^{-1} (data not shown) and attained equilibrium within 275 min at 23°C (Figure 2). At 37°C the binding was significantly reduced, attained equilibrium within 40 min, but was not stable and steadily declined between 100 and 210 min (Figure 3). Binding of 1 nM [^{3}H]$PGF_{2\alpha}$ was even lower than that of [^{3}H]AL-5848 at 37°C (Figure 3). Therefore, all subsequent studies were conducted at 23°C.

At 23°C, 5 nM [^{3}H]AL-5848 resulted in total binding of 1447 ± 24 disintegrations min^{-1} (n = 81) and non-specific binding of 226 ± 17 disintegrations min^{-1} (n = 81) (84% specific). At 37°C total binding was 576 ± 25 disintegrations min^{-1}

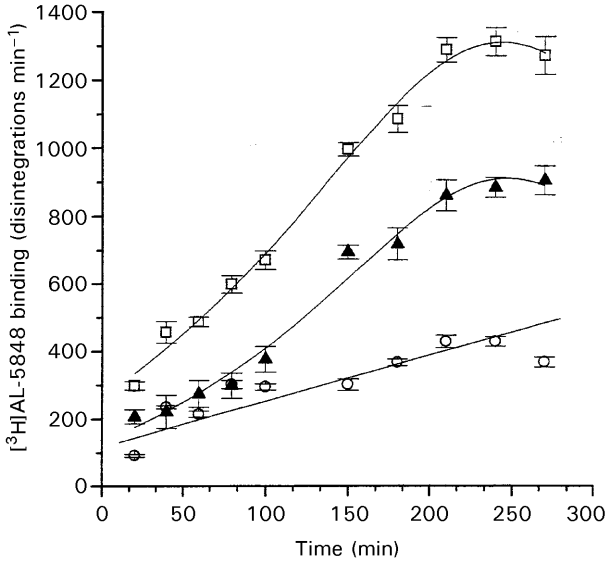

Figure 2. Time-course of [^{3}H]AL-5848 binding to washed total particulate bovine corpus luteum homogenates at 23°C. [^{3}H]AL-5848 (5 nM) binding was conducted in the absence (total, □) or presence (non-specific, ○) of $PGF_{2\alpha}$ (10 μM) for various times; ▲ indicates the specific binding. Data are means from a representative of several experiments; vertical lines show the s.e.m.

Table 1. Receptor-binding profile of AL-5848 (9-β-(+)-fluprostenol) and $PGF_{2\alpha}$ in PG receptor subtype binding assays.

Prostaglandin used	Prostaglandin receptor subtype binding affinity (K_i, nM) and selectivity relative to FP receptor affinity				
	DP	EP_3	FP	IP	TP
$PGF_{2\alpha}$	18 000 ± 6460 (150×)	24 ± 6 (−5×)	120 ± 9	> 50 000 (416×)	> 190 000 (1583×)
AL-5848	46 000 ± 5700 (885×)	3501 ± 461 (67×)	52 ± 10	> 90 000 (1713×)	> 121 000 (2327×)

Data are mean ± s.e.m. from 3–83 independent experiments. Selectivity relative to FP receptor affinity is shown in parentheses.

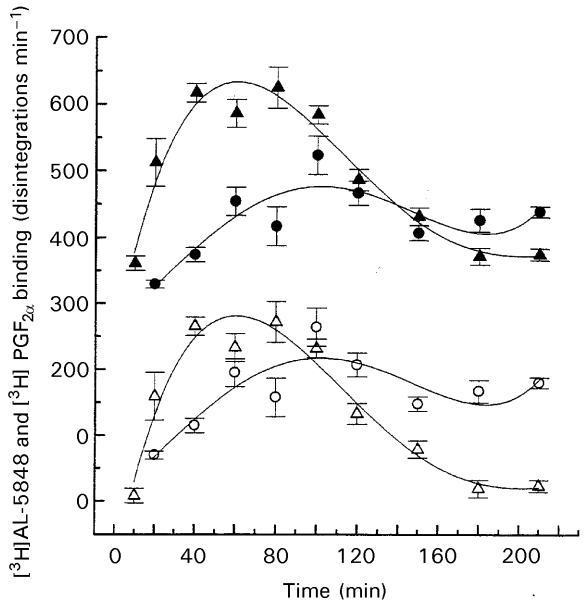

Figure 3. Time-course of [^{3}H]AL-5848 (5 nM; ▲, total; △, specific) and [^{3}H]PGF$_{2\alpha}$ (1 nM; ●, total; ○, specific) binding to washed total particulate bovine corpus luteum homogenates at 37°C. Data are means from a representative of several experiments; vertical lines show s.e.m.

(n = 34) and non-specific binding was 202 ± 18 (n = 34) (65% specific). Scatchard analysis of specific [^{3}H]AL-5848 binding obtained from saturation experiments indicated interaction with a single class of high-affinity (dissociation constant, K_d, = 33·8 ± 2·9 nM, n = 4) and saturable (B_{max} = 37·3 ± 3·0 pmol (g wet tissue)$^{-1}$ FP receptor binding sites (Figures 4 and 5). The equilibrium binding parameters were very similar when non-specific binding was defined with unlabelled (±)-fluprostenol or PGF$_{2\alpha}$ (Figures 4 and 5).

In competition experiments, specific [^{3}H]AL-5848 (5 nM) binding was concentration-dependently inhibited by a range of FP-receptor ligands with affinities (Figure 6; Table 2) commensurate with selective labelling of the FP-receptor binding sites in the bovine corpus luteum homogenates. The rank order of affinity of these FP-ligands was: 16-phenoxyPGF$_{2\alpha}$ > AL-5848 = cloprostenol > 17-phenylPGF$_{2\alpha}$ > (±)-fluprostenol > PGF$_{2\alpha}$ > PHX-A85 >>> S-1033 (Table 2). Specific [^{3}H]AL-5848 binding was minimally inhibited by PGD_2, ZK118182, BW245C, PGE_2, PGI_2, and U-46619 (K_i values > 1·5–407 μM; Table 2), ligands known to have a high affinity for non-FP receptors.

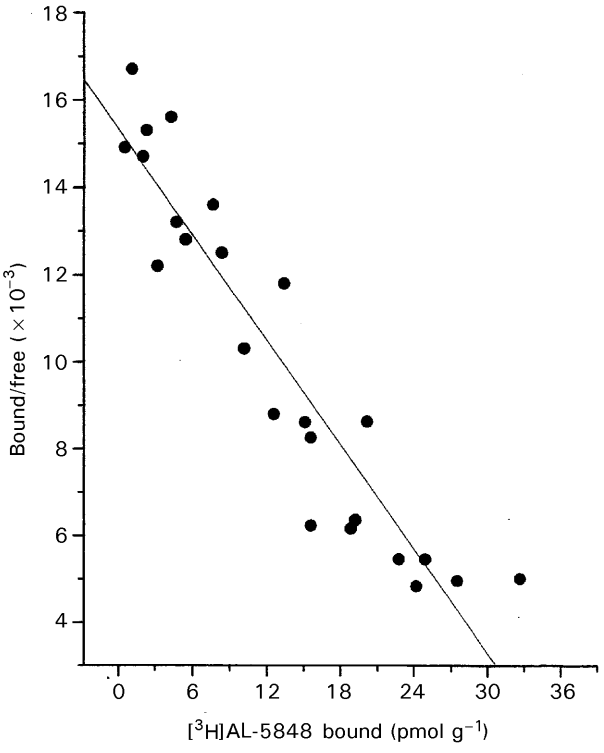

Figure 4. Scatchard plot of specific [^{3}H]AL-5848 binding to washed total particulate material in bovine corpus luteum homogenates at 23°C. Data are means of duplicate points from two experiments with 12 concentrations of [^{3}H]AL-5848 and using 10 μM unlabelled (±)-fluprostenol to define the non-specific binding. Similar results were obtained with 10 μM unlabelled PGF$_{2\alpha}$ (Figure 5).

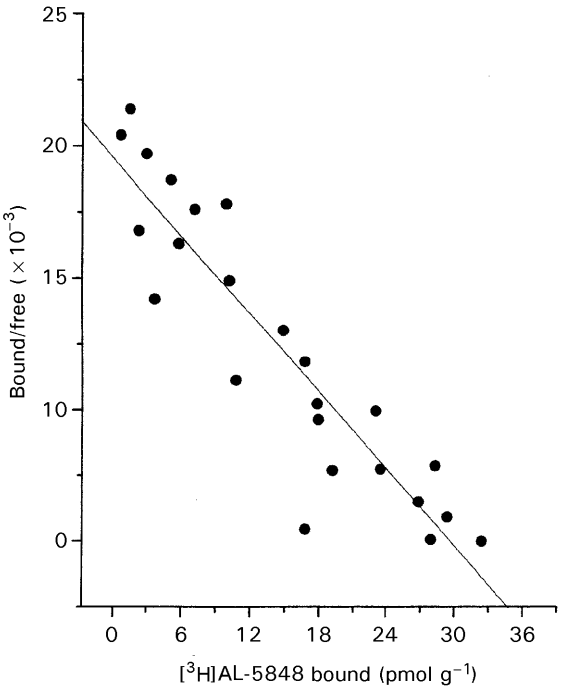

Figure 5. Scatchard plot of specific [^{3}H]AL-5848 binding to washed total particulate material in bovine corpus luteum homogenates at 23°C. Data are means of duplicates from two experiments with 12 concentrations of [^{3}H]AL-5848 and using 10 μM unlabelled PGF$_{2\alpha}$ to define the non-specific binding. Similar results were obtained with 10 μM unlabelled (±)-fluprostenol (Figure 4).

Table 2. Affinities of different prostaglandins for [^{3}H]AL-5848 and [^{3}H]$PGF_{2\alpha}$ binding to washed total particulate bovine corpus luteum homogenates.

Compound	Affinity (K_i, nM) relative to [^{3}H]AL-5848	$-\log K_i$ relative to [^{3}H]AL-5848	Affinity (K_i, nM) relative to [^{3}H]$PGF_{2\alpha}$	$-\log K_i$ relative to [^{3}H]$PGF_{2\alpha}$
16-Phenoxy$PGF_{2\alpha}$	17·2±2·9	7·7	22±5	7·66
AL-5848	52·1±2·6	7·28	52±10	7·28
Cloprostenol	56·8±6·3	7·14	31±3	7·51
17-Phenyl$PGF_{2\alpha}$	87±8	7·06	59±8	7·23
(±)-Fluprostenol	108±4·7	6·97	102±11	6·99
$PGF_{2\alpha}$	195±12	6·83	120±9	6·92
PHXA85	223±5	6·62	98±11	7·01
PGD_2	1500±122	5·87	2500±760	5·6
Latanoprost	3470±455	5·47	4200±790	5·38
PGE_2	8220±245	5·24	3400±710	5·47
U-46619	22 200±3560	4·66	8900±140	5·05
S-1033	28 300±2730	4·55	22 000±2600	4·66
ZK118182	103 000±18 400	4·13	69 000±19 000	4·16
PGI_2	268 000±98 500	3·63	86 000±29 000	4·06
BW245C	> 407 000	3·39	not determined	

Data are means ± s.e.m. from 3–11 experiments for [^{3}H]AL-5848 and from up to 50 experiments for [^{3}H]$PGF_{2\alpha}$. Experiments were conducted in duplicate at 23°C. The Hill coefficients for the competition curves were not significantly different from unity ($P > 0.05$).

The pharmacological characteristics of specific [^{3}H]AL-5848 binding detailed above and shown in Table 2 correlated well with the pharmacology of [^{3}H]$PGF_{2\alpha}$ binding to FP receptor sites in the bovine corpus luteum preparation ($r = 0.9$, $n = 14$, $P < 0.0001$) (Figure 7). There was also a good correlation between the affinities of 12 compounds competing for [^{3}H]AL-5848 binding at 23°C and 37°C ($r = 0.77$) (Figure 8). Likewise, receptor-binding affinities of a whole range of PGs against [^{3}H]AL-5848 binding and their agonist potencies at stimulating inositol phosphates generation in Swiss 3T3 and rat vascular smooth muscle cells (A7r5) were also well correlated ($r = 0.96$) (Figure 9).

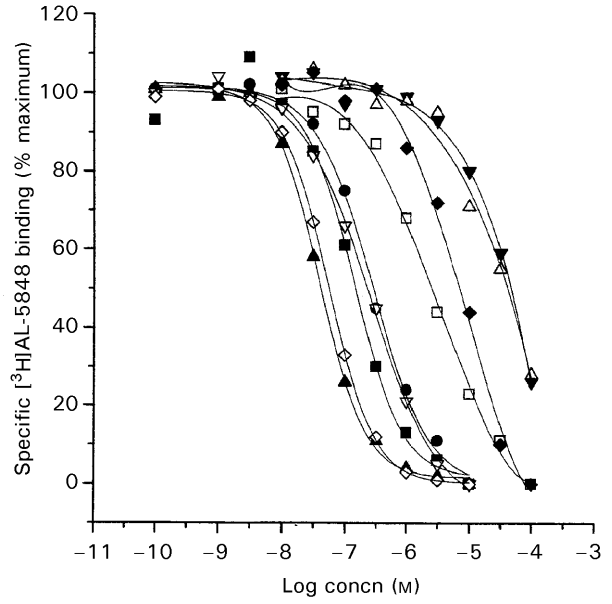

Figure 6. Competition curves for different prostaglandins displacing [^{3}H]AL-5848 binding from high-affinity FP receptor binding sites on washed total particulate bovine corpus luteum homogenates at 23°C. ▲ Cloprostenol, ◇ AL-5848, ■ (±)-fluprostenol, ▽ PHXA85, ● $PGF_{2\alpha}$, □ PGD_2, ◆ PGE_2, △ U-46619, ▼ S-1033. Data are means of duplicate determinations from one representative experiment. Composite data from 3–11 experiments are shown inTable 2.

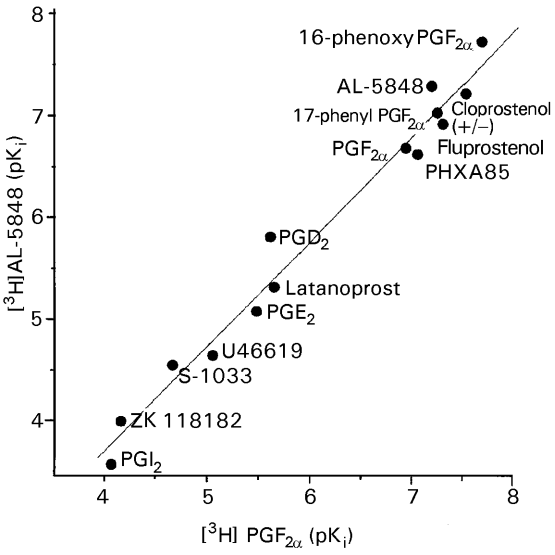

Figure 7. Correlation of the pharmacology of specific [^{3}H]AL-5848 and [^{3}H]$PGF_{2\alpha}$ binding to washed total particulate bovine corpus luteum homogenates at 23°C ($r = 0.988$; $P < 0.0001$).

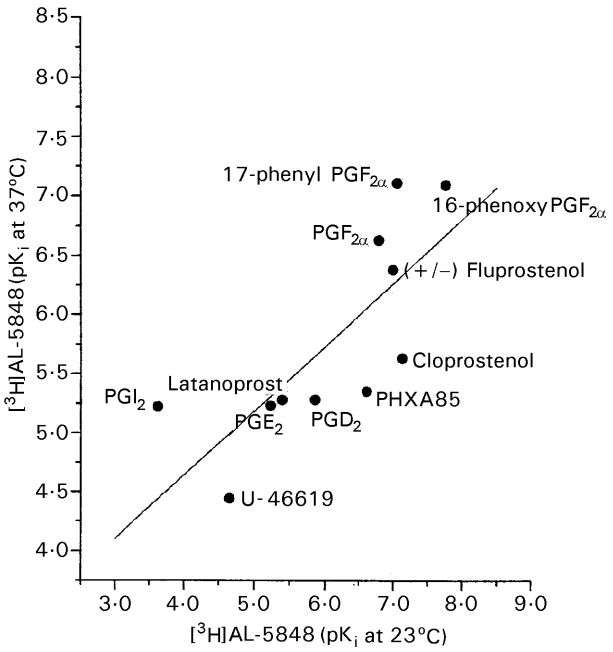

Figure 8. Correlation of the pharmacology of specific [^{3}H]AL-5848 binding to washed total particulate in bovine corpus luteum homogenates at 37°C and 23°C ($r = 0{\cdot}77$; $P < 0{\cdot}01$).

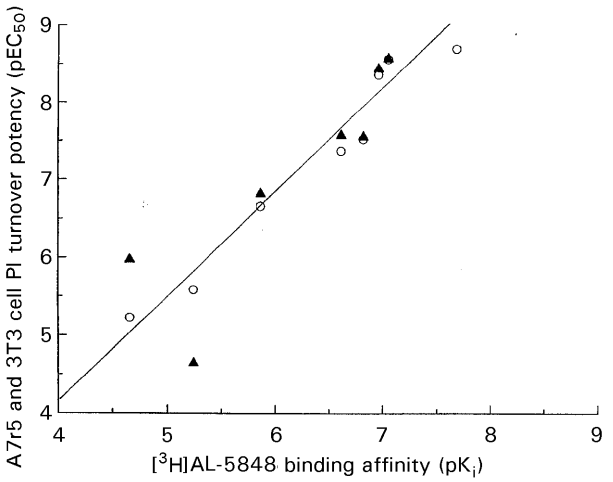

Figure 9. Correlation of the pharmacology of specific [^{3}H]AL-5848 binding to washed total particulate material in bovine corpus luteum homogenates and FP-receptor-mediated phosphatidylinositol (PI) turnover in A7r5 and 3T3 cells. Functional data for mouse Swiss 3T3 fibroblasts and A7r5 rat vascular smooth muscle cells were taken from Griffin et al (1997, 1998), respectively. ○ Swiss 3T3 cell data, ▲ A7r5 cell data ($r = 0{\cdot}96$; $P < 0{\cdot}01$).

In quantitative autoradiographic studies conducted on bovine corpus luteum and sections from eyes from man, high specific binding of [^{3}H]AL-5848 was associated with the granulosa cells in the bovine corpus luteum and in the longitudinal ciliary muscle, the ciliary process, the iris sphincter muscle and the retina in the eye (Figure 10; Table 3).

Table 3. Quantitative autoradiographic distribution of FP receptors by use of [^{3}H]AL-5848.

Tissue region	Specific [^{3}H]AL-5848 binding (digital light units mm^{-2})
Eye from man	
Iris sphincter muscle	14 367 ± 1325
Iris (minus sphincter)	13 261 ± 1187
Retina	3611 ± 1182
Ciliary epithelium/process	3593 ± 673
Lens	2628 ± 698
Longitudinal ciliary muscle	2517 ± 236
Circular ciliary muscle	1861 ± 352
Cornea	966 ± 371
Bovine corpus luteum	
Granulosa cells	115 212 ± 5154
Connective tissue and blood vessels	3268 ± 534

Eyes from human donors and bovine corpus luteum sections were radiolabelled with 40 nM [^{3}H]AL-5848. The specific binding in each region of interest was determined from several sections and donors by quantitative image analysis of the phosphor-imager screens. Data for the eye structures are mean ± s.e.m. from six different donors. The data for bovine corpus luteum are mean ± s.e.m. from >6 readings for each structure from up to six different sections. Specific binding ranged between 15–19% (e.g. cornea, lens) and 50–91% (e.g. iris sphincter of eye, granulosa cells of bovine corpus luteum) of the total in the regions shown above.

Discussion

The radiolabelling and pharmacological characterization of FP-receptor binding sites has historically been performed with the natural ligand [^{3}H]PGF$_{2\alpha}$. However, because of the relatively limited specificity of PGF$_{2\alpha}$ (Csukas et al 1993; Coleman et al 1994; Sharif et al 1998) and its limited stability, it is not an ideal radioligand. In these studies, AL-5848 [(9-β-(+)-fluprostenol)] was synthesized and shown to be an FP-receptor agonist with relatively high FP-receptor affinity and functional activity. Furthermore, [^{3}H]AL-5848 was generated and shown to bind to a single population of high-affinity FP-receptor binding sites in washed total particulate homogenates of bovine corpus luteum, a tissue which is very rich in FP receptors (Chegini et al 1991; Sharif et al 1998) and from which the first FP receptor was cloned (Sakamoto et al 1994). [^{3}H]AL-5848 binding to bovine corpus luteum homogenates was of high-affinity and saturable, indicative of labelling of specific FP-receptor binding sites in this tissue. The dissociation constant for [^{3}H]AL-5848 binding (K_d approx. 34 nM) compared well with that observed for [^{3}H]PGF$_{2\alpha}$ binding ($K_d = 21–50$ nM) to luteal membranes (Kimball & Lauderdale 1975; Hammarstrom et al 1976). However, in our studies a greater percentage

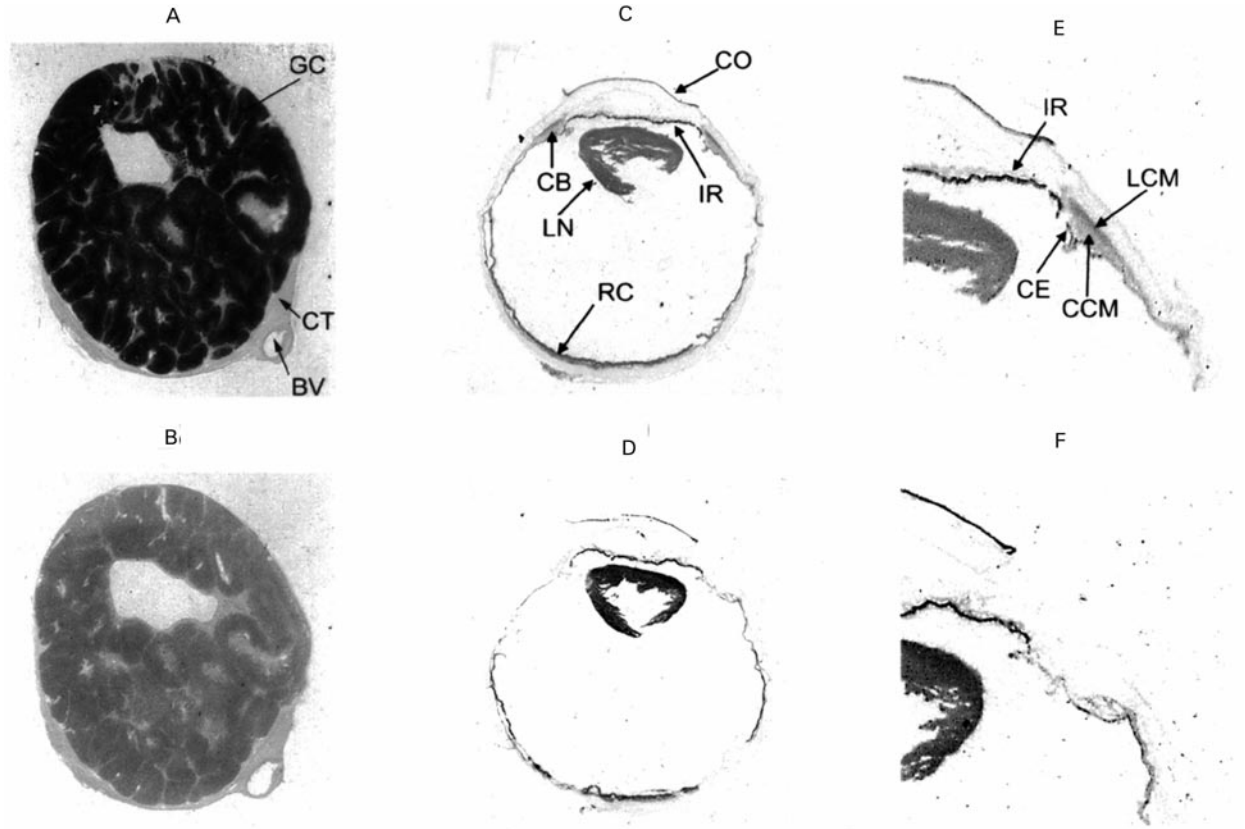

Figure 10. Autoradiographic location of FP-receptor-binding sites in bovine corpus luteum and sections of eye from man. Panels A and B show FP-receptor labelling in bovine corpus luteum sections with 40 nM [^{3}H]AL-5848, with A representing total and B representing non-specific binding. Panels C and D show total and non-specific binding in sections of the eye from a 62-year-old donor at low power and panels E and F show the anterior chamber structures of interest at higher magnification. The low-power images in panels A–D were taken at 1·75× magnification of the original; panels E and F are at 4·7× magnification of the original. GC = granulosa cell, CT = connective tissue, BV = blood vessel, CO = cornea, CB = ciliary body, IR = iris, LN = lens, RC = retina-choroid, LCM = longitudinal circular muscle, CCM = circular ciliary muscle, CE = ciliary epithelium.

of specific binding was found for [^{3}H]AL-5848 than for [^{3}H]PGF$_{2\alpha}$, especially when assayed at 37°C, perhaps emphasizing the greater stability of [^{3}H]AL-5848 than [^{3}H]PGF$_{2\alpha}$. It is noteworthy that optimum radioligand binding to FP receptors present on tissue homogenates in-vitro occurs at 22–23°C rather than at 37–38°C or 0–4°C (Kimball & Lauderdale 1975; Wright et al 1979), these being the conditions used also in our present studies.

The pharmacological characteristics of binding of [^{3}H]AL-5848 to bovine corpus luteum homogenates was indicative of labelling of FP-receptor binding sites. Hence, well known FP-receptor agonists, for example cloprostenol, PHXA85 (latanoprost acid) and 17-phenylPGF$_{2\alpha}$ (Coleman et al 1994; Abramovitz et al 1995) competed for [^{3}H]AL-5848 binding with nanomolar affinities, whereas ligands with some selectivity for DP (e.g. ZK118182, BW245C), EP (PGE$_2$), IP (PGI$_2$) and TP (U46619) receptors had micromolar affinities. It was worthy of note that the rank order and overall correlation of the pharmacology of [^{3}H]AL-5848 binding compared well with the pharmacology of [^{3}H]PGF$_{2\alpha}$ binding (Figure 7) and that of the potencies of various PGs in stimulating phosphatidylinositol turnover in Swiss 3T3 mouse fibroblasts and rat vascular smooth muscle cells (A7r5) (Figure 9). This overall pharmacological profile of [^{3}H]AL-5848 binding was also similar to that recently described for [^{3}H]PGF$_{2\alpha}$ binding to bovine corpus luteum membranes (Goh & Kishino 1994; Sharif et al 1998), except that [^{3}H]AL-5848 is a more FP-receptor-selective radioligand.

The similarity of autoradiographic labelling of FP-receptors in bovine corpus luteum sections and in sections of eyes from man with [^{3}H]AL-5848 (this study) and that reported recently using [^{3}H]PGF$_{2\alpha}$ in bovine corpus luteum (Chegini et al 1991; Sharif et al 1998) and in post-mortem eyes from man (Matsuo & Cynader 1992; Davis & Shariff 1999) also attests to the labelling of FP receptor sites in these tissues with these radioligands. However, the more specific binding, and the greater stability and specificity of [^{3}H]AL-5848 over [^{3}H]PGF$_{2\alpha}$, should make [^{3}H]AL-5848 the radioligand of choice for future work. The high

FP-receptor labelling with [^{3}H]AL-5848 in the granulosa cells in the bovine corpus luteum is because FP-receptor-mediated phosphatidylinositol turnover, intracellular calcium mobilization and hormone release occurs via these cells in this tissue (Davis et al 1987; Niswender & Nett 1988; Chegini et al 1991). In terms of the ocular location of [^{3}H]AL-5848- and [^{3}H]PGF$_{2\alpha}$-labelled FP receptors, it is known that ligands in the FP-class partially reduce intraocular pressure by promoting aqueous humour outflow (Toris et al 1993) by modulating the tension of the longitudinal ciliary muscle (Wang et al 1990; Goh et al 1995) and partially by increasing extracellular matrix degradation (Weinreb et al 1997) via intracellular calcium mobilization (Chen et al 1997). FP agonists also influence the smooth muscle activity of the iris in various animals (Yousufzai et al 1996; Bito 1997) and the iridial pigmentation observed with the FP agonist latanoprost (Stjernschantz et al 1995; Bito 1997), might be because of activation of FP receptors in the iris (Bito 1997). There is also evidence for the involvement of the FP receptor in the iris and ciliary muscles in the mediation of the release of other prostaglandins (Abdel-Latif 1995). Our autoradiographic location of FP-receptor sites in eyes from man using [^{3}H]AL-5848 also correlated well with recent data reported from in-situ hybridization and immunohistochemical studies of FP receptors conducted on cynomolgus monkey eyes in-vitro (Ocklind et al 1996) and with the ocular effects of FP agonists mentioned above, including the reduction of the intraocular pressure in glaucomatous cynomolgus monkey eyes by the isopropyl ester of AL-5848 (AL-6221) (Hellberg et al 1998). The level of specific binding in the eye structures was lower than that associated with the granulosa cells in the bovine corpus luteum, because the overall FP receptor density is lower in the eye and the presence of melanin in most ocular regions leads to higher non-specific binding. Similar observations have previously been made with [^{3}H]PGF$_{2\alpha}$-binding to sections of eye from man (Matsuo & Cynader 1992; Davis & Sharif 1999).

The data presented above indicate that [^{3}H]AL-5848 is an FP-receptor-selective PG with a reasonably high affinity which labels an homogeneous population of FP-receptor sites in the bovine corpus luteum with pharmacological features akin to those previously reported for FP receptors in this and other cells and in other tissues (Coleman et al 1994). The apparent superior specificity and stability of [^{3}H]AL-5848 over [^{3}H]PGF$_{2\alpha}$ should make [^{3}H]AL-5848 a useful radioligand for receptor-binding and autoradiographic studies on various biological tissues to enable better understanding of the physiology and pharmacology of the FP receptor.

Acknowledgements

We thank Drs Jesse May, Robert Selliah and Paul Zinke in the Medicinal Chemistry Department for their synthetic work on the prostaglandins used in the current studies. The authors are grateful for the critical review of the manuscript by Drs Tom Dean and Mark Hellberg and other Alcon internal reviewers and their support and encouragement of these studies. The donors and their families who made the ocular autoradiographic studies possible are gratefully acknowledged.

References

Abdel-Latif, A. A. (1995) Phosphoinositide and arachidonic acid signalling systems in the mammalian iris. Prog. Retinal Eye Res. 14: 75–107

Abramovitz, M., Adam, M., Boie, Y., Grygorczyk, R., Rushmore, T. H., Funk, C. D., Bastien, L., Sawyer, N., Rochette, C., Sliptz, D. M., Metters, K. M. (1995) Human prostanoid receptors: cloning and characterization. Adv. Prostaglandins Thromb. Leuk. Res. 23: 499–504

Andersen, N. H., Ramwell, P. W. (1974) Biological aspects of prostaglandins. Arch. Intern. Med. 133: 30–50

Armstrong, R. A., Lawrence, R. A., Jones, R. L., Wilson, N. H., Collier, A. (1989) Functional and ligand binding studies suggest heterogeneity of platelet prostacyclin receptors. Br. J. Pharmacol. 97: 657–668

Bito, L. Z. (1997) Prostaglandins: a new approach to glaucoma management with a new intriguing side-effect. Surv. Ophthalmol. 41 (Suppl. 22): S1–S14

Blue, D. R., Bonhaus, D., Ford, A. P. D., Pfister, J., Sharif, N. A., Shieh, A., Vimont, R., Williams, T. J., Clarke, D. J. (1995) Functional evidence equating the pharmacologically-defined α_{1A}- and cloned α_{1C}-adrenoceptors: studies in the isolated perfused kidney of rat. Br. J. Pharmacol. 115: 283–294

Bowen, W. P., Jerman, J. (1995) Nonlinear regression using spreadsheets. Trends Pharmacol. Sci. 16: 413–417

Chegini, N., Lei, Z. M., Rao, C. V., Hansel, W. (1991) Cellular distribution and cycle phase dependency of gonadotropin and eicosanoid binding sites in bovine corpora lutea. Biol. Reproduction 45: 506–513

Chen, J., Champa-Rodriguez, M. L., Woodward, D. F. (1995) Identification of a prostanoid FP receptor population producing endothelium-dependent vasorelaxation in the rabbit jugular vein. Br. J. Pharmacol. 116: 3035–3041

Chen, W., Andon, T., Bhattacherjee, P., Paterson, C. (1997) Intracellular calcium mobilization following prostaglandin receptor activation in human ciliary muscle cells. Curr. Eye Res. 16: 847–853

Cheng, Y. C., Prusoff, W. H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC50) of an enzymatic reaction. Biochem. Pharmacol. 22: 3099–3108

Coleman, R. A., Smith, W. L., Narumiya, S. (1994) VIII. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol. Rev. 46: 205–229

Cooper, B., Ahern, D. (1979) Characterization of the platelet prostaglandin D_2 receptor. J. Clin. Invest. 64: 586–590

Csakas, S., Bhattacherjee, P., Rhodes L., Paterson, C. A. (1993) Prostaglandin E_2 and $F_{2\alpha}$ binding sites in the bovine iris ciliary body.. Invest. Opthalmol. Vis. Sci. 34: 2237–2245

Davis, J. S., Weakland, L. L., Weiland, D. A., Farese, R. V., West, L. A. (1987) Prostaglandin $F_{2\alpha}$ stimulates phosphatidylinositol 4,5- bisphosphate hydrolysis and mobilizes intracellular Ca^{2+} in bovine luteal cells. Proc. Natl Acad. Sci. USA 84: 3728–3732

Davis, T. L., Sharif N. A. (1999) Quantitative autoradiographic visualization and pharmacology of FP-prostaglandin receptors in human eyes using the novel phosphor-imaging technology. J. Ocular Pharmacol. Ther. (in press)

Fernandes, B., Crankshaw, D. (1995) Functional characterization of the DP receptor in human myometrium. Eur. J. Pharmacol. 283: 73–81

Goh, Y., Kishino, J. (1994) Pharmacological characterization of prostaglandin-related ocular hypotensive agents. Jpn J. Ophthalmol. 38: 236–245

Goh, Y., Hotehama, Y., Mishima, H. K. (1995) Characterization of ciliary muscle relaxation induced by various agents in cats. Invest. Ophthalmol. Vis. Sci. 36: 1188–1192

Griffin, B. W., Williams, G. W., Crider, J. Y., Sharif, N. A. (1997) FP prostaglandin receptors mediating inositol phosphate generation and calcium mobilization in Swiss 3T3 cells: a pharmacological study. J. Pharmacol. Exp. Ther. 281: 845–854

Griffin, B. W., Magnino, P. E., Pang, I.-H., Sharif, N. A. (1998) Pharmacological characterization of an FP prostaglandin receptor on rat vascular smooth muscle cells (A7r5) coupled to phosphoinositide turnover and intracellular calcium mobilization. J. Pharmacol. Exp. Ther. 286: 411–418

Hammarstrom, S., Powell, W. S., Kylden, U., Samuelsson, B. (1976) Some properties of a prostaglandin $F_{2\alpha}$ receptor in corpora lutea. In: Samuelsson, B., Paoletti, R. (eds) Advances in Prostaglandin and Thromboxane Research, Vol. 1, Raven Press, New York, pp 235–246

Hellberg, M., Sallee, V., Mclaughlin, M., Sharif, N. A., Desantis, L. M., Dean, T. R., Zinke, P. (1998) Preclinical efficacy of AL-6221, a potent and selective FP prostaglandin agonist. Invest. Ophthalmol. Vis. Sci. 39 (Suppl. 4): Abstr. 1961

Kimball, F. A., Lauderdale, J. W. (1975) Prostaglandin E_1 and $F_{2\alpha}$ specific binding in bovine corpora lutea: comparison with luteolytic effects. Prostaglandins 10: 313–331

Matsuo, T., Cynader, M. S. (1992) Localization of prostaglandin $F_{2\alpha}$ and E_2 binding sites in the human eye. Br. J. Ophthalmol. 76: 210–213

McPherson, G. A. (1983) A practical computer based approach to the analysis of radioligand binding experiments. Computer Prog. Biomed. 17: 107–114

Narumiya, S. (1994) Prostanoid receptors: structure, function, and distribution. In: Goetzl, E. J., Lewis, R. A., Rola-Pleszczynski, M. (eds) Cellular Generation, Transport, and Effects of Eicosanoids: Biological Roles and Pharmacological Intervention, The New York Academy of Sciences, New York, pp 126–138

Niswender, G. D., Nett, T. M. (1988) Corpus luteum and its control. In: Knobll, E., Neil, J. (eds) The Physiology of Reproduction, Raven Press, New York, pp 489–525

Ocklind, A., Lake, S., Wentzel, P., Nister, M., Sternschantz, L. (1996) Localization of the prostaglandin $F_{2\alpha}$ receptor messenger RNA and protein in the cynomolgus monkey eye. Invest. Ophthalmol. Vis. Sci. 37: 716–726

Ogletree, M. L., Allen, G. T. (1992) Interspecies differences in thromboxane receptors: studies with thromboxane receptor antagonists in rat and guinea pig smooth muscles. J. Pharmacol. Exp. Ther. 260: 789–794

Sakamoto, K., Ezashi, T., Miwa, K., Okuda-Ashitaka, E., Houtani, T., Sugimoto, T., Ito, S., Hayaishi, O. (1994) Molecular cloning and expression of a cDNA of the bovine prostaglandin $F_{2\alpha}$ receptor. J. Biol. Chem. 269: 3881–3886

Sallee, V., Mclaughlin, M., Griffin, B. W., Sharif, N. A. (1998) Correlation of results from preclinical experimental models used for evaluation of FP prostaglandin agonists for therapy of glaucoma. Invest. Ophthalmol. Vis. Sci. 39 (Suppl. 4): Abstr. 4274

Senior, J., Marshall, K., Sangha, R., Clayton, J. K. (1993) In vitro characterization of prostanoid receptors on human myometrium at term pregnancy. Br. J. Pharmacol. 108: 501–506

Sharif, N. A. (1996) Quantitative autoradiographic methods. In: Toga, A. W., Mazziota, J. C. (eds), Brain Mapping: The Methods, Academic Press, New York, pp 115–144

Sharif, N. A., Hughes, J. (1989) Discrete mapping of brain mu and delta opioid receptors using selective peptides: quantitative autoradiography, species differences and comparison with kappa receptors. Peptides 10: 499–522

Sharif, N. A., Wong, E. H. F., Loury, D., Stefanich, E., Eglen, R. M., Michel, A. D., Whiting, R. L. (1991) Characteristics of $5HT_3$ binding sites in rat cerebral cortex, NG108-15 and NCB-20 neuroblastoma cells using [^{3}H]quipazine and [^{3}H]GR65630 binding. Br. J. Pharmacol. 102: 919–925

Sharif, N. A., Xu, S. X., Williams, G. W., Crider, J. Y., Griffin, B. W., Davis, T. L. (1998) Pharmacology of [^{3}H]prostaglandin E_1/[^{3}H]prostaglandin E_2 and [^{3}H]prostaglandin $F_{2\alpha}$ binding to EP_3 and FP receptor binding sites in bovine corpus luteum: characterization and correlation with functional data. J. Pharmacol. Exp. Ther. 286: 1094–1102

Stjernschantz, J., Selen, G., Sjoquist, B., Resul, B. (1995) Preclinical pharmacology of latanoprost, a phenyl-substituted $PGF_{2\alpha}$ analogue. Adv. Prostaglandin. Thrombox. Leukotr. Res. 23: 513–518

Toris, C. B., Camras, C. B., Yablonski, M. E. (1993) Effects of PhXA41, a new prostaglandin $F_{2\alpha}$ analog, on aqueous humour dynamics in human eyes. Ophthalmol. 100: 1297–1304

Upham, L. V., Englert, D. F. (1998) Radionuclide imaging. In: L'Lannunziata, M. (ed.) Handbook of Radioactivity Analysis, Academic Press, New York, pp 647–692

Wang. R.-F., Camras, C. B., Lee, P.-Y., Podos, S. M., Bito, L. Z. (1990) Effects of prostaglandins $F_{2\alpha}$, A_2, and their esters in glaucomatous monkey eyes. Invest. Ophthalmol. Vis. Sci. 31: 2466–2470

Weinreb, R. N., Kashiwagi, K., Kashiwagi, F., Tsukahara, S., Lindsey, J. D. (1997) Prostaglandins increase matrix metalloprosteinase release from human ciliary smooth muscle cells. Invest. Ophthalmol. Vis. Sci. 38: 2770–2772

Wright, K., Luborsky-Moore, J. L., Behrman, H. R. (1979) Specific binding of prostaglandin $F_{2\alpha}$ to membranes of rat corpora lutea. Mol. Cell. Endocrinol. 13: 25–34

Yousufzai, S. Y. K., Ye, Z., Abdel-Latif, A. A. (1996) Prostaglandin $F_{2\alpha}$ and its analogs induce release of endogenous prostaglandins in iris and ciliary muscles isolated from cat and other mammalian species. Exp. Eye Res. 63: 305–310