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RESEARCH PAPER

Identification of an antagonist that selectively blocks the activity of prostamides (prostaglandin-ethanolamides) in the feline iris

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Background and Purpose: The prostamides (prostaglandin-ethanolamides) and prostaglandin (PG) glyceryl esters are biosynthesized by COX-2 from the respective endocannabinoids anandamide and 2-arachidonyl glycerol. Agonist studies suggest that their pharmacologies are unique and unrelated to prostanoid receptors. This concept was further investigated using antagonists.
Experimental Approach: The isolated feline iris was used as a key preparation, where prostanoid FP receptors and prostamide activity co-exist. Activity at human recombinant FP and other prostanoid receptors was determined using stable transfectants.
Key Results: In the feline iris, AGN 204396 produced a rightward shift of the dose-response curves for prostamide $F_{2\alpha}$ and the prostamide $F_{2\alpha}$ analog bimatoprost but did not block the effects of $PGF_{2\alpha}$ and synthetic FP receptor agonists. Studies on human recombinant prostanoid receptors confirmed that AGN 204396 did not behave as a prostanoid FP receptor antagonist. AGN 204396 exhibited no antagonism at DP and $EP_{1\text{-}4}$, but was a highly effective TP receptor antagonist. Contrary to expectation, the FP receptor antagonist AL-8810 efficaciously contracted the cat iris. AGN 204396 did not affect AL-8810 induced contractions, demonstrating that AL-8810 and AGN 204396 are pharmacologically distinct. Unlike AL-8810, the ethylamide derivate of AL-8810 was not an agonist. Al-8810 did not block prostamide $F_{2\alpha}$ activity. Finally, AGN 204396 did not block PGE_2-glyceryl ester activity.
Conclusions and Implications: The ability of AGN 204396 to selectively block prostamide responses suggests the existence of prostamide sensitive receptors as entities distinct from receptors recognizing $PGF_{2\alpha}$ and PGE_2-glyceryl ester.
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Keywords: prostaglandins; prostglandin ethanolamides; FP receptor; iris; prostaglandin-glyceryl ester

Abbreviations: AAALAC, Association of Assessment and Accreditation of Laboratory Animal Care,International; 2-AG, 2-arachidonyl glycerol; ARVO, Association for Research in Vision and Ophthalmology; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; CR, concentration–response; Cyr 61, cysteine-rich angiogenic protein; EBNA, Epstein–Barr nuclear antigen; ELISA, enzyme-linked immunosorbent assay; FAAH, fatty acid amide hydrolase; FLIPR, fluorimetric imaging plate reader; HA, hemagglutanin; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*-[2-ethanesulphonic acid]; HRP, horseradish peroxidase; PG, prostaglandin; 15-OH PGDH, 15-OH prostaglandin-dehydrogenase; PKC, prostein kinase C; PPARγ, peroxisome proliferation-activated receptor γ; USDA, United States Department of Agriculture

Introduction

The endocannabinoids arachidonyl ethanolamide (anandamide) and 2-arachidonyl glyceryl ester (2-AG) are substrates for cyclooxygenase-2 (COX-2), with resultant conversion to the corresponding prostaglandin (PG) ethanolamides and glyceryl esters. This COX-2-specific biosynthetic pathway leads to the formation of a spectrum of PG ethanolamides (prostamides) and glyceryl esters that closely approache the diversity of the prostanoids (Yu *et al*., 1997; Burstein *et al*., 2000; Kozak *et al*., 2002; Koda *et al*., 2003). The biological significance of this pathway remains to be elucidated, but initial studies using recombinant enzymes have transitioned into cell (Kozak *et al*., 2002; Glass *et al*., 2005) and living animal studies (Weber *et al*., 2004).

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A number of suggestions have been advanced and investigated to account for the biological significance of the PG ethanolamides (prostamides) and glyceryl esters. These include cannabimimetics (Berglund *et al*., 1999), vanilloid receptor agonists (Matias *et al*., 2004), prostaglandin mimetics (Sharif *et al*., 2001), fatty acid amide hydrolase (FAAH) substrates that indirectly reduce anandamide metabolism (Matias *et al*., 2004) and activators of peroxisome proliferation-activated receptor γ (PPARγ) (Rockwell and Kaminski, 2004). The greatest emphasis has been placed on comparative pharmacological studies with PGs, which suggest that the PG ethanolamides and glyceryl esters are pharmacologically unique. Thus, the effects of PGE_2 ethanolamide in the guinea-pig trachea could not be readily explained by interaction with prostanoid EP receptors (Ross *et al*., 2002). Similarly, the Ca^{2+} signal and induction of protein kinase C (PKC) activity produced by PGE_2 glyceryl ester were independent of conversion to PGE_2 and did not appear to involve PGE_2-sensitive receptors (Nirodi *et al*., 2004). The most extensive pharmacological studies to date have been performed on $PGF_{2\alpha}$-ethanolamide (prostamide $F_{2\alpha}$) and its structural analogue bimatoprost. As in the case of PGE_2-glyceryl ester, the effects prostamide $F_{2\alpha}$ and bimatoprost appear unrelated to PG formation and PG receptor stimulation and the existence of a population of receptors that preferentially recognize these molecules has been proposed (Woodward *et al*., 2001, 2003; Liang *et al*., 2003; Matias *et al*., 2004; Chen *et al*., 2005; Spada *et al*., 2005).

By virtue of its clinical status, bimatoprost is the most studied prostamide at this point in time. Bimatoprost behaves as a prostamide mimetic and is also the most efficacious antiglaucoma agent reported from patient studies to date (Dubiner *et al*., 2001; Higginbotham *et al*., 2002; Noecker *et al*., 2003; Parrish *et al*., 2003; Woodward *et al*., 2004). Moreover, patients refractory to latanoprost therapy are successfully treated with bimatoprost (Gandolfi and Cimino, 2003), suggesting a pharmacological distinction at the clinical level between the prostamide analog bimatoprost and the prostanoid FP receptor agonist prodrug latanoprost. Bimatoprost exhibits potent inherent pharmacological activity in certain *in vitro* pharmacological systems. These preparations include contraction of the cat lung (Woodward *et al*., 2003), cat iris (Woodward *et al*., 2001) and rabbit uterus (Chen *et al*., 2005), and upregulation of cysteine-rich angiogenic protein (Cyr 61) in human ciliary smooth muscle cells (Liang *et al*., 2003). Like the prostamides, bimatoprost activity at wild-type and recombinant FP and other prostanoid receptors is residual and occurs only at concentrations above 10^{-6} M (Sharif *et al*., 2001; Kelly *et al*., 2003; Woodward *et al*., 2003; Matias *et al*., 2004; Chen *et al*., 2005).

To date, pharmacological characterization of the prostamides has relied on agonist studies. The main focus has been comparing the activities of prostamide $F_{2\alpha}$ and bimatoprost with those of $PGF_{2\alpha}$ and selective prostanoid FP receptor agonists in a variety of prostamide-sensitive and-insensitive preparations. In all but one of these previous studies (Spada *et al*., 2005), it was not entirely clear if prostamide-sensitive tissues expressed a receptor subpopulation that preferentially recognized prostamides or an FP receptor subtype that equally recognized prostamides, $PGF_{2\alpha}$ and FP receptor selective PG analogs. Resolution of this issue and further elucidation of prostamide receptor pharmacology demand the identification of an antagonist. This would allow the existing prostamide receptor hypotheses to be refuted or supported. With this aim, the effects of a recently discovered compound that blocked prostamide activity were compared to those of the FP receptor antagonist AL-8810 (Griffin *et al*., 1999) and its ethylamide derivative in the isolated feline iris. These studies suggest that there is a pharmacological distinction between prostamide and prostanoid FP receptor responses.

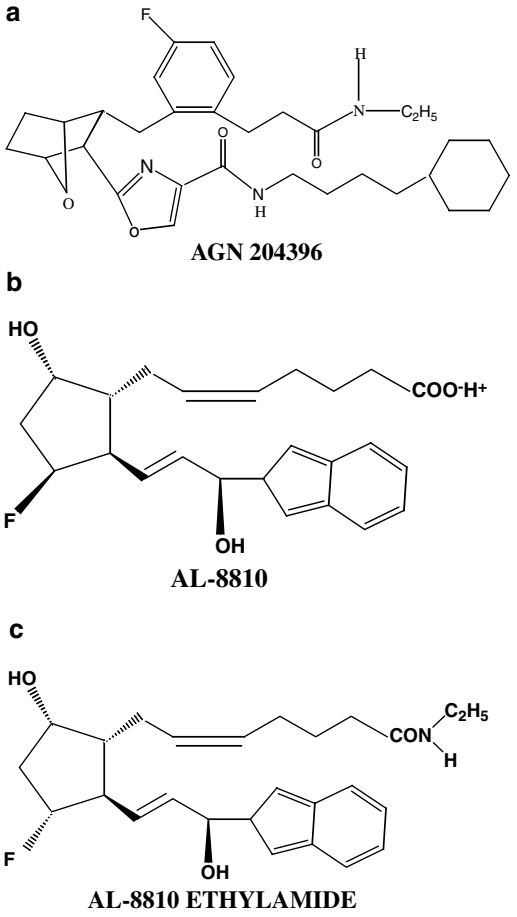

Figure 1 Structures of (**a**) AGN 204396, (**b**) AL-8810 and (**c**) AL-8810 ethylamide.

Methods

Test systems used

The test systems employed included the isolated feline iris as a key preparation as both prostamides (prostaglandin ethanolamides) and prostanoid FP receptor agonists potently elicit a contractile response in this tissue (Woodward *et al*., 2003; Matias *et al*., 2004). Activity at human prostanoid receptors was determined using recombinant receptors stably transfected into HEK-293 EBNA cells, using chimeric G proteins to enable Ca^{2+} signal responses to all receptor subtypes, as described previously (Woodward *et al*., 2003; Matias *et al*., 2004).

Measurements

Feline iris. Class A laboratory bred cats were housed communally in United States Department of Agriculture (USDA) and Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) approved facilities, with standards that exceeded those for enrichment and group housing. Water was available *ad libitum* and food was standard cat nutritional diet. They were kept on a 12-h light–dark cycle. They (96) were euthanized by intravenous (i.v.) overdose of sodium pentobarbital (Anthony, Arcadia, CA, USA). The eyes were enucleated immediately thereafter and placed on ice. Two eyes provided a total of four iridial preparations. The iris sphincter was mounted vertically under 50–100 mg tension in a jacketed 10 ml organ bath. Smooth muscle tension of the isolated iris sphincter was measured isometrically with force displacement transducers (Grass FT-03) and recorded on a Grass polygraph (Model 7). The organ baths contained Krebs' solution maintained at 37°C by a heat exchanger and circulating pump. The Krebs' solution was gassed with 95% O_2, 5% CO_2 to give a pH of 7.4, and had the following composition: 118.0 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.9 mM $CaCl_2$, 1.18 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 11.7 mM glucose and 0.001 mM indomethacin. A 60-min stabilization period was provided before commencing each experiment. Activity was manifest as contractile responses and measured as such. These investigations were as humane as possible and adhered to the 'Association for Research in Vision and Ophthalmology (ARVO) resolution on the Use of Animals in Research'.

Ca^{2+} signal studies on human recombinant prostanoid receptors. The use of chimeric G protein cDNAs allowed responses to G_s-and G_i-coupled prostanoid receptors to be measured as a Ca^{2+} signal, as described previously (Woodward *et al*., 2003; Matias *et al*., 2004). Prostanoid DP, EP_2 and EP_4 receptor cDNAs were co-transfected with chimeric G_{qs} cDNA containing a hemagglutanin (HA) epitope. The prostanoid EP_3 receptor was co-transfected into HEK-293 EBNA cells, using $pCEP_4$ as a vector, with chimeric G_{qi}-HA. G_{qs} and G_{qi} chimeric cDNAs (Molecular Devices, Sunnyvale, CA, USA) were cloned into a $pCEP_4$ vector and also selected by using a hygromycin B selection marker. Transfection into HEK-293

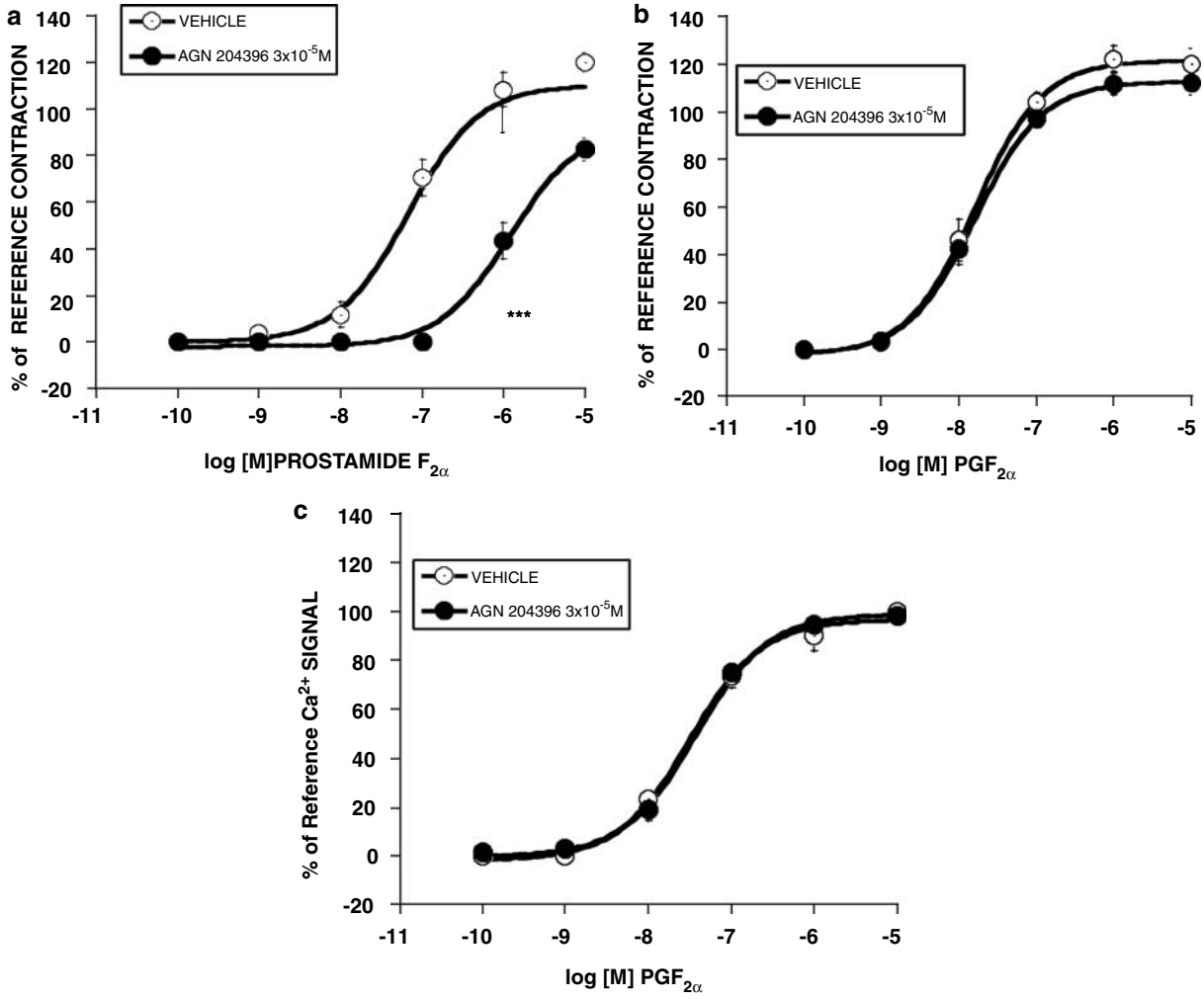

Figure 2 Effects of AGN 204396 (3×10^{-5} M) on contraction of the feline iris produced by (**a**) prostamide $F_{2\alpha}$ (**b**) $PGF_{2\alpha}$ and on (**c**) $PGF_{2\alpha}$ induced Ca^{2+} signaling in stable transfectants expressing human recombinant FP receptors. Values are mean ± s.e.m.; (**a**), (**b**) $n = 6$ and (**c**) $n = 3$ of duplicate determinations. ***$P < 0.0001$ comparing EC_{50} values.

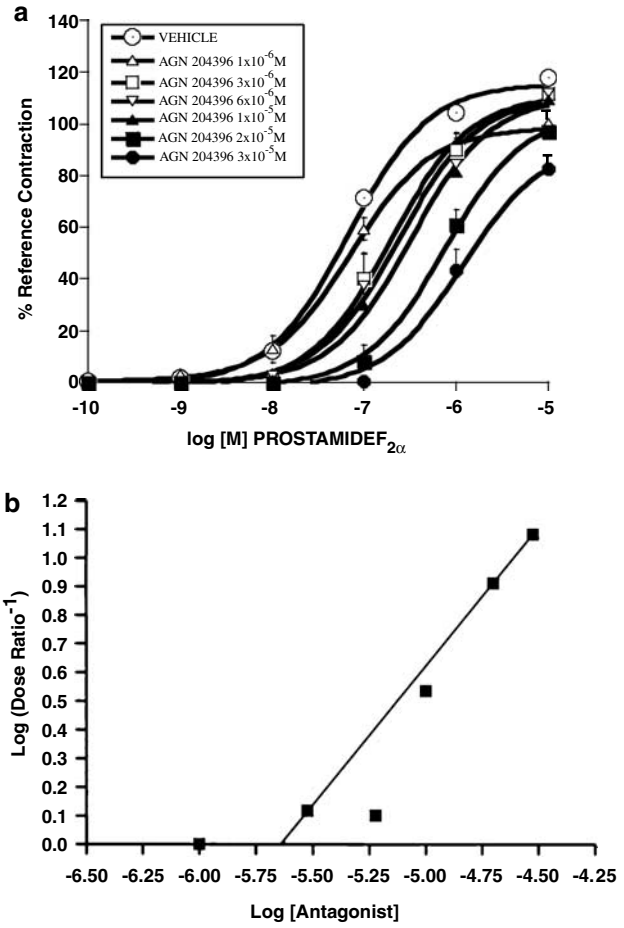

Figure 3 Effects of graded doses of AGN 204396 on contraction of the feline iris produced by prostamide $F_{2\alpha}$ (**a**). Points for the vehicle-treated iris represent the mean ± s.e.m. of the mean values for each of the six individual antagonist experiments. Values are mean ± s.e.m.; $n = 6$ for each concentration of AGN 204396. A Schild plot of these data is depicted in (**b**).

EBNA cells was achieved by the FuGENE 6 method. Because G_{qs} and G_{qi} contained an HA epitope, protein expression was detected by Western blotting analysis using anti-mouse HA monoclonal antibody and horseradish peroxidase (HRP)-conjugated secondary antibody. For human recombinant EP_1, FP, IP and TP receptors, stable transfectants were obtained as described previously (Woodward *et al*., 2003; Matias *et al*., 2004). Briefly, $pCEP_4$ was used as the expression vector and transfection into HEK-293-EBNA cells was performed with FuGENE 6. Stable transfectants were again selected according to hygromycin resistance.

Ca^{2+} signaling studies were performed using a fluorimetric imaging plate reader (FLIPR) instrument. Cells were seeded at a density of 5×10^4 cells/well in Biocoat poly-D-lysine-coated, black wall, clear bottom 96-well plates (BD Biosciences, Franklin Lakes, NJ, USA) and allowed to attach overnight in an incubator at 37°C. The cells were then washed twice with Hanks' balanced salt solution (HBSS)–*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulphonic acid] (HEPES) buffer (Hanks' balanced salt solution without bicarbonate and phenol red, 20 mM HEPES, pH 7.4) using a Denley Cellwash plate washer (Labsystems, Franklin, MA, USA). After 45–60 min of dye loading in the dark using the Ca^{2+}-sensitive dye Fluo-4AM, at a final concentration of 2×10^{-6} M, the plates were washed four times with HBSS–HEPES buffer to remove excess dye and leaving 100 μl of buffer in each well. The plates were then placed in the FLIPR instrument and allowed to equilibrate at 37°C. Compound solutions were added in a 50-μl volume to each well to give the desired final concentration. Cells were excited with an argon laser at 488 nm and emission was measured through a 510–570 nm bandwidth emission filter (FLIPR, Molecular Devices, Sunnyvale, CA, USA). The peak increase in fluorescence intensity was recorded for each well.

Experimental design

The feline iris experiments were designed so that a direct, four-way comparison for antagonist vs prostamide, vehicle vs prostamide, antagonist vs corresponding PG, and vehicle vs corresponding PG was provided in tissue preparations obtained from a single animal. One cumulative dose–response curve to agonist was obtained in each tissue. Vehicle (ethanol) and antagonist (AGN 204396) were given 30 min before the agonist dose–response curves were constructed. The response to $PGF_{2\alpha}$ 10^{-7} M was determined at the beginning and end of each dose–response curve, with appropriate washout, and responses were calculated as % of this reference contraction.

The experimental design for the FLIPR studies was as follows. On each plate, four wells each served as negative (HBSS–HEPES buffer) and positive controls (standard agonist: DP = BW 245C, EP_1–EP_4 = PGE_2, FP = $PGF_{2\alpha}$, IP = carbaprostacyclin, TP = U-46619). The peak fluorescence change in each well containing drug was expressed relative to the controls. To obtain concentration–response curves, compounds were tested in duplicate in each plate over the desired concentration range. Each compound was tested on at least three separate plates using cells from different passages to give $n = 3$.

Data analysis and statistical procedures

In order to calculate the pA_2 value for AGN 204396 in the feline iris preparation, the mean concentration–response curve was plotted as log concentration–response −1 (CR−1) vs log antagonist concentration according to the method of Arunlakshana and Schild (1959), using GraphPad Prism 4 software. As sequential use of graded doses of AGN 204396 with washout in a single tissue was impossible (AGN 204396 is lipophilic and very difficult to washout), an adaptation of the prostamide $F_{2\alpha}$ concentration–response curve in the presence of vehicle was required for analysis. Thus, for analysis, each point on the prostamide $F_{2\alpha}$ vs vehicle concentration-response curve represents the mean of each of the vehicle vs prostamide $F_{2\alpha}$ responses from the six separate antagonist experiments. As the slope of the CR−1 vs log[Antagonist] plot did not significantly differ from unity, the slope was constrained to 1. The effects of antagonists for all other experiments were statistically analyzed by comparing the midpoints of the curves ($\log EC_{50}$'s) for the agonist

concentration–response curves in the presence or absence of antagonist on the basis of an *F*-test. Null or alternative hypotheses were rejected at a minimum 0.05 level. All data were analyzed using GraphPad Prism 4.

Materials

AGN 204396, 3-(2-{(1*R*,2*R*,3*S*,4*R*)-3-[4-(4-cyclohexyl-butylcarbamoyl)-oxazol-2-yl]-7-oxa-bicyclo[2.2.1] hept–2-ylmethyl)-4-fluoro-phenyl)-propyl ethylamide, was synthesized at Allergan, Inc. (Irvine, CA, USA) Prostaglandins D_2, E_2 and $F_{2\alpha}$, U-46619, BW245C, AL-8810 were purchased from Cayman Chemical (Ann Arbor, MI, USA). AL-8810 ethylamide was synthesized by Selcia Ltd (Ongar, UK). Prostaglandin D_2, E_2 and $F_{2\alpha}$ were synthesized at Allergan, Inc. or purchased from Cayman Chemical (Ann Arbor, MI, USA). Prostaglandin E_2 1-glyceryl ester was a gift from LJ Marnett (Vanderbilt University, Nashville, TN, USA). All stock solutions were prepared in ethanol.

Results

The structure of the prostamide antagonist AGN 204396, and AL-8810 and its ethanolamide derivative are depicted in Figure 1.

The effects of AGN 204396, 3×10^{-5} M, on contractions produced by prostamide $F_{2\alpha}$ and $PGF_{2\alpha}$ are shown in Figure 2. AGN 204396 produced a rightward shift of the prostamide $F_{2\alpha}$ concentration–response curve (Figure 2a) but no meaningful displacement of the $PGF_{2\alpha}$ concentration–response curve in the feline iris (Figure 2b) or the $PGF_{2\alpha}$ concentration–response curve for Ca^{2+} signaling in human recombinant FP receptors (Figure 2c) was obtained. Comparing the mid-points (log EC_{50}'s) of the concentration–response curves at the maximum responses attained in the presence or absence of AGN 204396, the prostamide $F_{2\alpha}$ response was antagonized at the $P<0.0001$ significance level but $PGF_{2\alpha}$ responses were not significantly altered. The effects of graded concentrations of AGN 204396 on prostamide $F_{2\alpha}$-induced iridial contraction are shown in Figure 3a. A concentration-dependent rightward displacement was apparent, with the 10^{-6} M concentration being essentially ineffective. A Schild analysis is provided in Figure 3b. The points were distributed within the linear range of slope = 1. The pA_2 was 5.64. It was noticeable that the effects of AGN 204396 were never totally surmounted by 10 μM prostamide $F_{2\alpha}$. This may be due to residual activity at FP receptors (Matias *et al*., 2004), which are present in the feline iris (Coleman *et al*., 1984; Spada *et al*., 2005). The effects of AGN 204396 as an antagonist on bimatoprost-evoked iridial contraction is depicted in Figure 4a, both 10^{-5} and 3×10^{-5} M concentrations were effective. Although AGN 204396 blocked feline iridial contraction produced by the prostamide analog bimatoprost at the $P<0.0001$ significance level, it did not significantly affect the response to 17-phenyl $PGF_{2\alpha}$ (Figure 4b). The effects of AGN 204396 on iridial contraction produced by latanoprost free acid and fluprostenol are shown in Figure 5, no significant antagonism was apparent.

The effects of a 3×10^{-5} M concentration of AGN 204396 on Ca^{2+} signals associated with human recombinant prostanoid receptor stimulation are depicted in Figure 6.

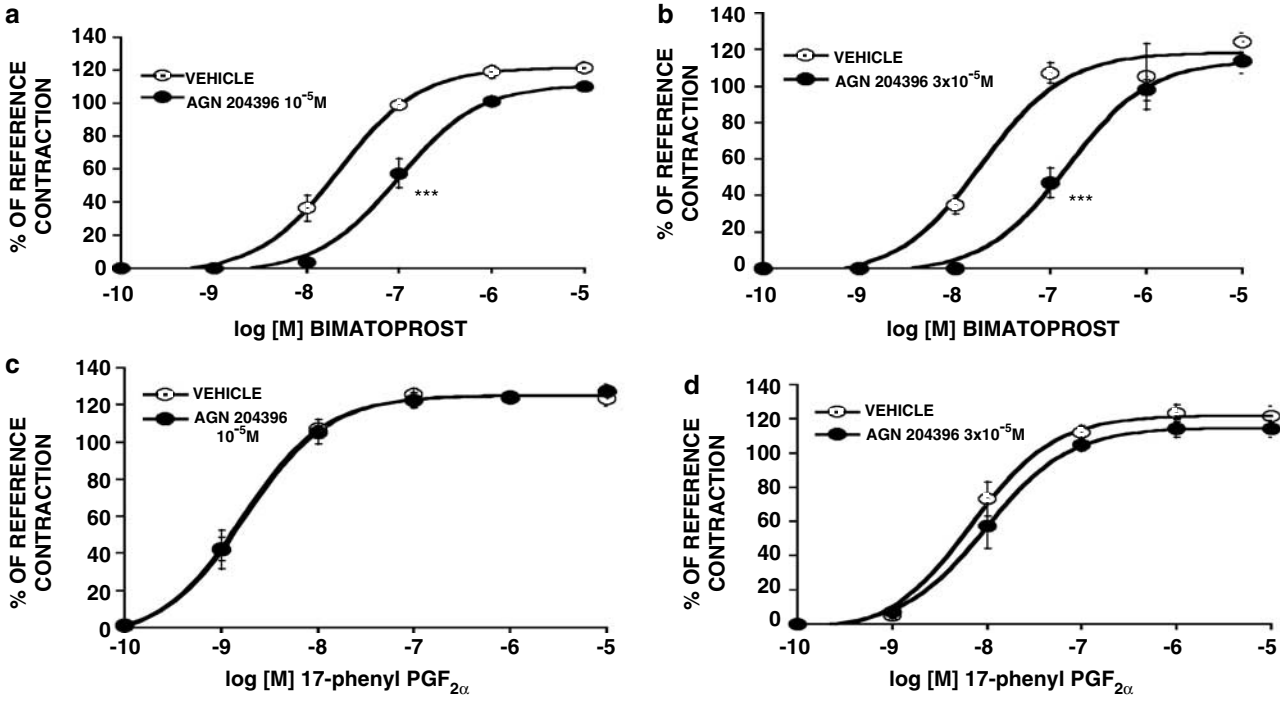

Figure 4 Effects of AGN 204396 on contraction of the feline iris produced by bimatoprost and 17-phenyl $PGF_{2\alpha}$. The effects of AGN 204396 at 10^{-5} and 3×10^{-5} M on bimatoprost-induced contractions are shown (**a**) and (**b**), respectively. The effects of AGN 204396 at 10^{-5} and 3×10^{-5} M on 17-phenyl $PGF_{2\alpha}$-induced contraction are shown (**c**) and (**d**), respectively. Values are mean ± s.e.m.; $n=6$ for 3×10^{-5} M AGN 204396, $n=12$ for 10^{-5} M AGN 204396. ***$P<0.0001$ comparing EC_{50} values.

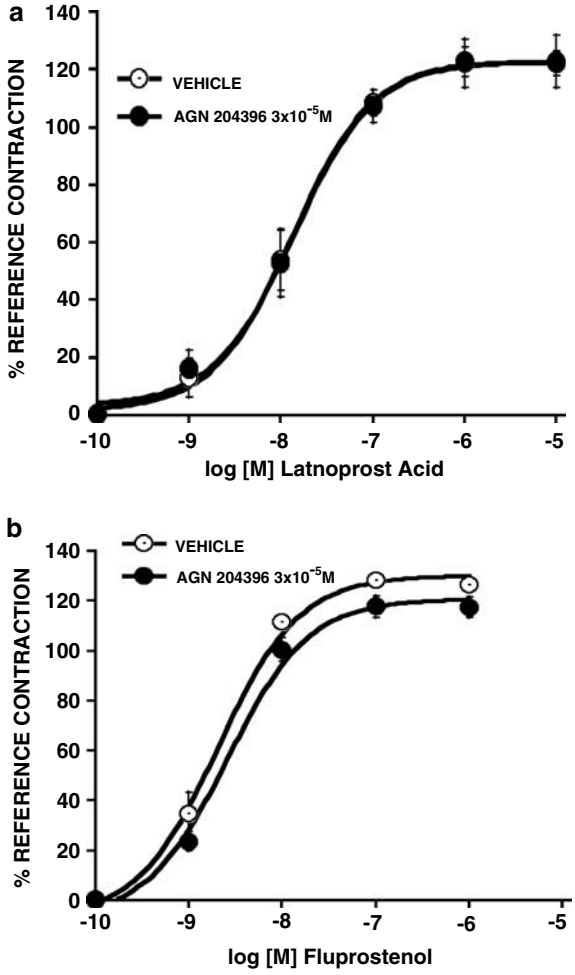

Figure 5 Effects of AGN 204396 (3×10^{-5} M) on feline iridial contraction produced by (**a**) latanoprost free acid and (**b**) fluprostenol. Values are mean ± s.e.m.; $n = 6$.

No antagonism was apparent at DP, EP_{1-4} or FP receptors, but AGN 204396 was a highly efficacious TP antagonist (Figure 6), with a k_B value of 1.49×10^{-8} M. It was also a very weak IP receptor antagonist and appeared to potentiate PGE_2 activity at the EP_1 receptor, which was quite unexpected.

The effects of AGN 204396 on iridial contraction produced by prostamide D_2 and E_2 and the corresponding free acids PGD_2 and PGE_2 are graphically depicted in Figure 7. AGN 204396 exhibited clear and significant antagonistic activity vs prostamide D_2 (Figure 7a) and prostamide E_2 (Figure 7b). This was similar to activity recorded vs prostamide $F_{2\alpha}$. AGN 204396 was much less active against PGD_2 (Figure 7c) and PGE_2-induced (Figure 7d) iridial contraction, but there was a modest and statistically significant shift in the PGE_2 concentration–response curve.

An attempt to use AL-8810 as an FP receptor antagonist (Griffin *et al*., 1999) was made in the feline iris. Contrary to expectation, AL-8810 appeared to behave as a weak, but full agonist in the feline iris preparation. To provide further pharmacological elucidation, a decision was made to attempt to block the effects of AL-8810 with AGN 204396. The iridial contraction produced by AL-8810 was not affected by AGN 204396 (Figure 8). This result indicates that AL-8810 and AGN 204396 are pharmacologically distinct. The ethylamide derivative of AL-8810 was also synthesized and tested. This did not contract the cat iris at concentrations up to 10^{-4} M. Moreover, AL-8810 ethylamide did not significantly antagonize prostamide $F_{2\alpha}$ effects (Figure 9).

PGE_2 1-glyceryl ester has recently emerged as a unique and interesting biologically active substance (Nirodi *et al*., 2004). AGN 204396 did not block the effect of PGE_2 1-glyceryl ester in the feline iris, suggesting that prostamide and PGE_2 1-glyceryl esters effects are distinct. These data are shown in Figure 10. Statistical comparisons of the effects of both AGN 204396 and AL-8810 ethylamide on contraction of the feline iris produced by various PGs, prostamides, and their analogs are provided in Table 1. An identical comparison for the antagonist effects of AGN 204396 3×10^{-5} M at human recombinant prostanoid receptors is given in Table 2.

Discussion and conclusions

Previous studies on PG-ethanolamides (prostamides) have suggested that they may produce their effects by interacting with receptive targets that are distinct from PG receptors (Woodward *et al*., 2001; Ross *et al*., 2002; Matias *et al*., 2004). These present studies were performed to extend earlier investigations and employed a compound that selectively attenuated prostamide activity. The isolated feline iris sphincter was selected for these studies as it is very responsive to both prostamide $F_{2\alpha}$ and prostanoid FP receptor agonists (Coleman *et al*., 1984; Matias *et al*., 2004), thereby providing an exacting test of the prostamide receptor hypothesis. The feline iris is also advantageous in that Ca^{2+} signals in response to the prostamide analog bimatoprost occur in a different cell population to those cells that respond to $PGF_{2\alpha}$ and the FP receptor agonist 17-phenyl $PGF_{2\alpha}$ (Spada *et al*., 2005). This offered an encouraging basis for the possible pharmacological separation of prostamide and FP receptor-mediated activities in the feline iris by intervention with putative antagonists. The antagonists selected for study were the FP receptor antagonist AL-8810 (Griffin *et al*., 1999), the ethylamide derivative of AL-8810 and a novel compound that appeared to block prostamide effects, designated AGN 204396.

AGN 204396 selectively antagonized the effects of prostamide $F_{2\alpha}$ and its analog bimatoprost in the feline iris but did not similarly affect responses to $PGF_{2\alpha}$ and prostanoid FP receptor selective agonists. AGN 204396 also antagonized the effects of prostamide D_2 and prostamide E_2 but was much less active in inhibiting the activities of the corresponding PGs, namely PGD_2 and PGE_2. Consistent with its lack of meaningful antagonist activity on PG-induced iridial contraction, AGN 204396 did not exhibit antagonist activity at human recombinant DP, EP_1, EP_2, EP_3, EP_4 or FP receptors. AGN 204396 was a potent TP receptor blocker but this activity does not interfere with analysis of the feline iris experiments, as this preparation does not contain functional TP receptors (Coleman *et al*., 1984) and the prostamides are not TP agonists (Matias *et al*., 2004). Similar reasoning applies to the very weak effect to AGN 204396 vs prostanoid

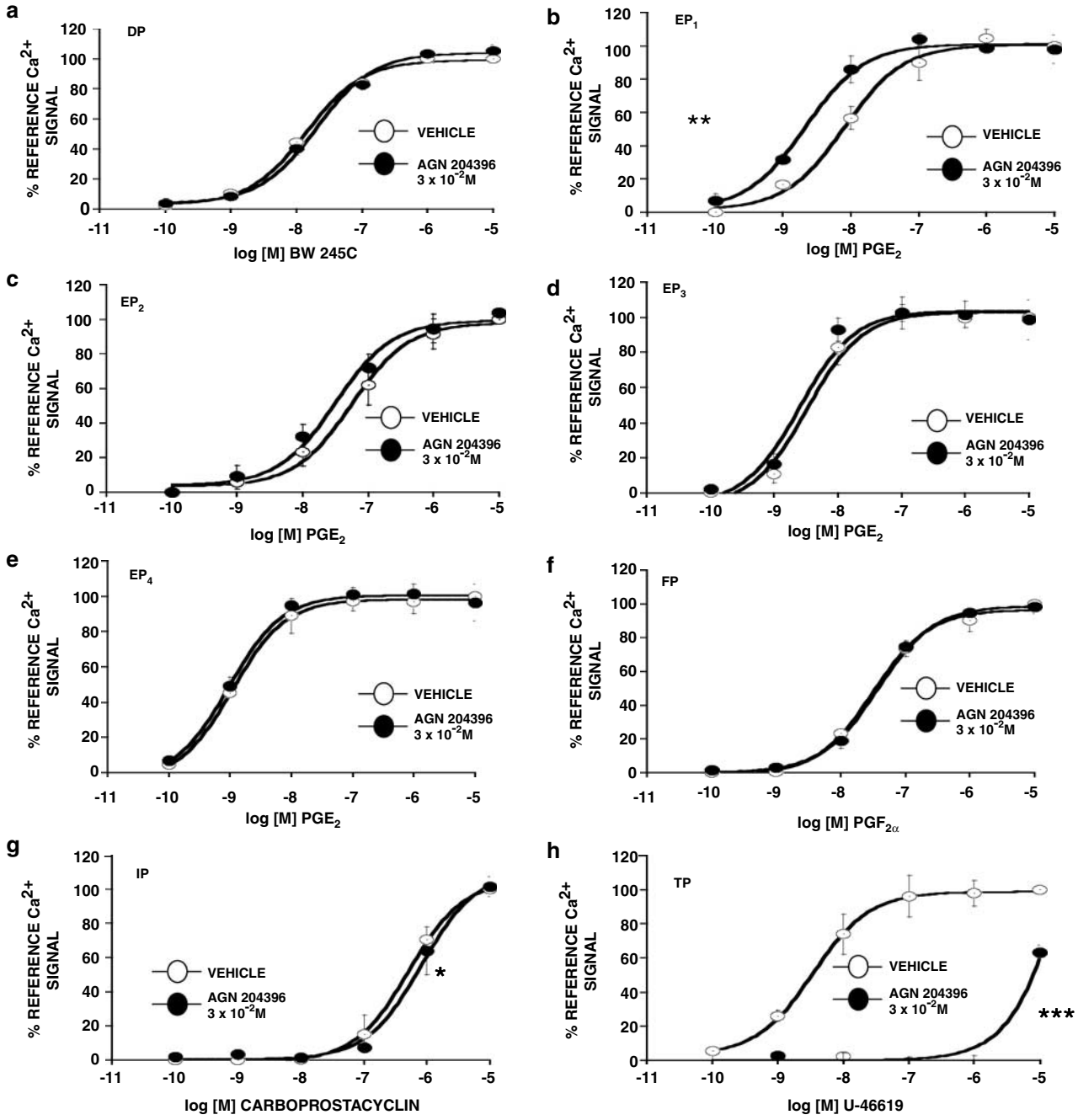

Figure 6 Effects of AGN 204396 (3×10^{-5} M) on Ca^{2+} signals associated with human recombinant prostanoid receptors stably transfected in HEK-293 cells. Antagonism at (**a**) DP, (**b**) EP_1, (**c**) EP_2 (**d**) EP_3, (**e**) EP_4, (**f**) FP, (**g**) IP and (**h**) TP receptors was evaluated. Values are mean ± s.e.m.; $n = 3$ of duplicate determinations. $^*P<0.05$, $^{**}P<0.01$ $^{***}P<0.001$ comparing EC_{50} values.

IP receptors. From these data, AGN 204396 appears to behave as a compound uniquely capable of selectively blocking prostamide activity. Taken together with previous data obtained with prostamide $F_{2\alpha}$ and its structural analog bimatoprost (Liang *et al*., 2003; Woodward *et al*., 2003; Matias *et al*., 2004; Chen *et al*., 2005; Spada *et al*., 2005), the results obtained with AGN 204396 are consistent with the hypothesis that prostamides may interact with a population of target receptors that is distinct in some way from known prostanoid receptors.

In addition to the prostamide antagonist studies, we also attempted 'mirror-image' studies to block $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ effects with the prostanoid FP receptor antagonist AL-8810 (Griffin *et al*., 1999). Contrary to expectation, AL-8810 exhibited efficacious myotropic activity in the feline iris sphincter. Such activity was not anticipated as agonist effects of AL-8810 have been found to be minimal or absent in previous cell and isolated tissue studies (Griffin *et al*., 1999; Hutchinson *et al*., 2003; Liang *et al*., 2004). Nevertheless, residual FP receptor stimulation is apparent for

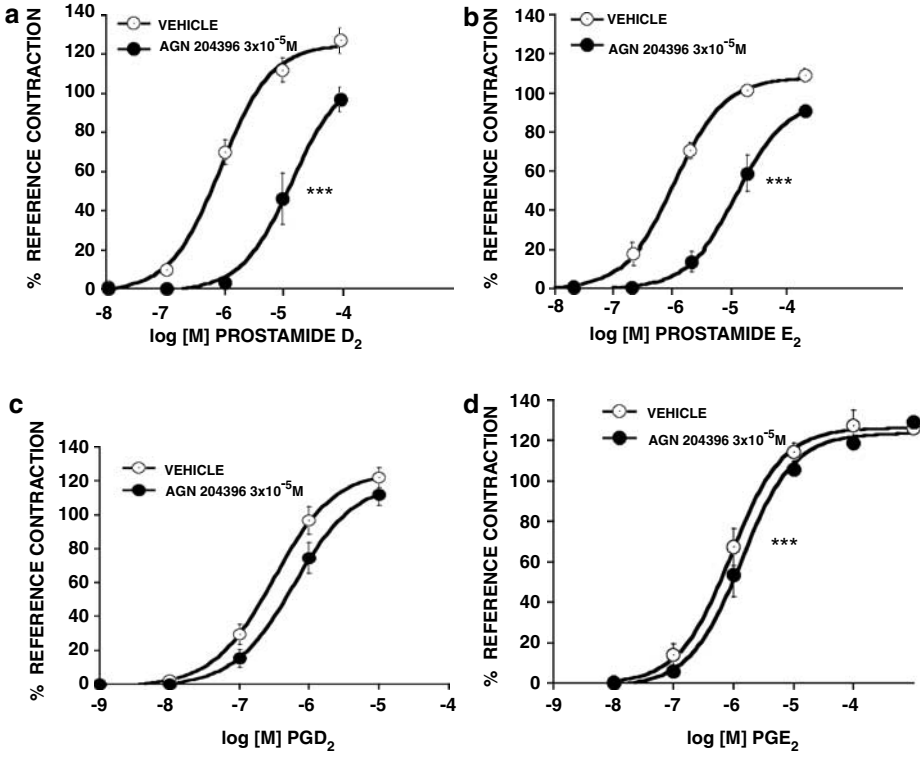

Figure 7 Effects of AGN 204396 (3×10^{-5} M) on contraction of the feline iris in response to (**a**) prostamide D_2 (**b**) prostamide E_2 (**c**) PGD_2 and (**d**) PGE_2. Values are mean ± s.e.m.; $n = 6$. ***$P < 0.0001$ comparing EC_{50} values.

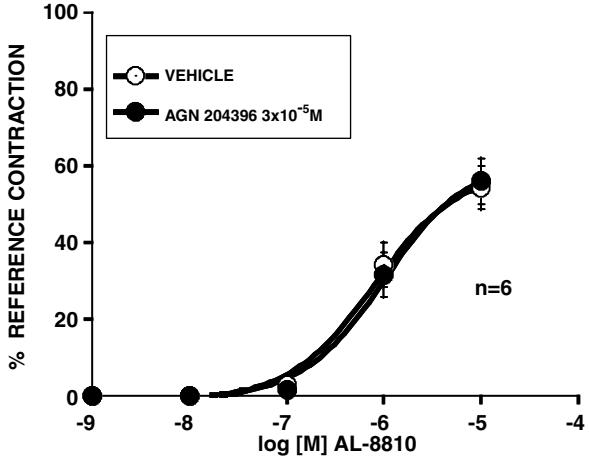

Figure 8 Effect on AGN 204396 (3×10^{-5} M) on contraction of the feline iris produced by AL-8810. Values are mean ± s.e.m.; $n = 6$.

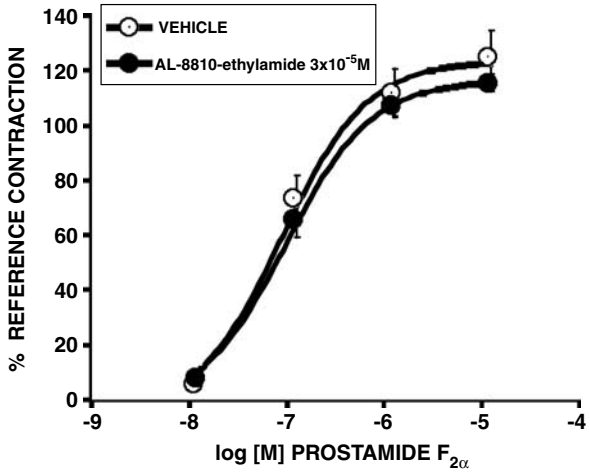

Figure 9 Effect of AL-8810 ethylamide (3×10^{-5} M) on contraction of the feline iris produced by prostamide $F_{2\alpha}$. Values are mean ± s.e.m.; $n = 6$.

Al-8810 in A7r5 cells (Griffin *et al*., 1999), which suggests partial agonism . It seems likely that AL-8810 is stimulating FP receptors in the feline iris given the following: (a) it can weakly agonize the FP receptor (Griffin *et al*., 1999; Hutchinson *et al*., 2003), (b) Al-8810 is a close structural analog of $PGF_{2\alpha}$, (c) An FP receptor has been functionally identified in the cat iris by the present study and the original prostanoid receptor classification (Coleman *et al*., 1984). As AL-8810 was unsuitable for use as an FP receptor antagonist in the feline iris, alternative experiments to those originally envisaged were undertaken. Thus, it was decided to examine the effect of AGN 204396 on AL-8810-induced iridial contraction to reveal any pharmacological interaction. Responses to AL-8810 were unaffected by AGN 204396 pretreatment, further indicating that the activity of AGN 204396 is not directly related to prostanoid FP receptors. Synthetic conversion of AL-8810 to its ethylamide derivative produced a compound that did not contract the feline iris at concentrations up to 10^{-4} M and did not block the effects of

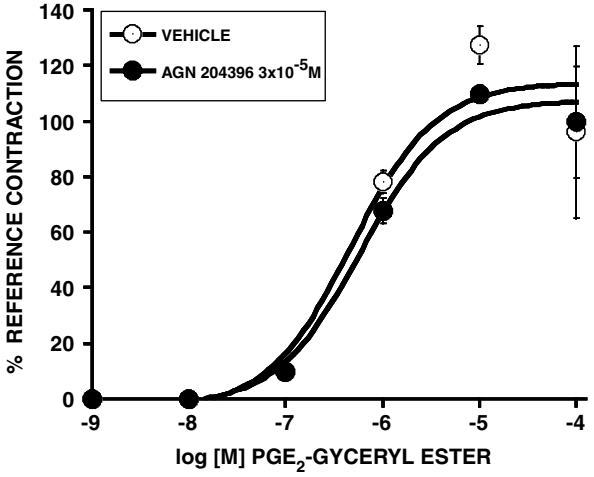

Figure 10 Effect of AGN 204396 (3×10^{-5} M) on contraction of the feline iris in response to PGE_2 1-glyceryl ester. Values are mean ± s.e.m.; $n = 6$.

prostamide $F_{2\alpha}$. This result indicates that the structural requirements for FP receptor and prostamide receptor antagonism are not identical, thereby providing a further point of differentiation for prostanoid and prostamide pharmacology. Finally, the absent biological activity of AL-8810 ethylamide indicates that conversion to the biologically active free acid (AL-8810) does not occur. This is consistent with studies demonstrating that prostamide $F_{2\alpha}$ and bimatoprost exert their activities as the intact molecules, according to direct analytical and bioassay-based metabolism studies (Woodward *et al*., 2003; Matias *et al*., 2004; Chen *et al*., 2005).

In addition to anandamide, a further endocannabinoid 2-AG is a substrate for COX-2, with the resultant biosynthesis of PG-glyceryl esters (Kozak *et al*., 2001, 2002). PGE_2 1-glyceryl ester was reported to mobilize intracellular Ca^{2+} and activate PKC by a mechanism that did not involve PGE_2 formation or PGE_2-sensitive receptors (Nirodi *et al*., 2004). As PGE_2 1-glyceryl ester also appears to be pharmacologically

Table 1 Antagonist activity of AGN 204396 at various concentrations and AL-8810 ethylamide at 3×10^{-5} M in the feline iris

Agonist vs antagonist identity	*Agonist log EC50 ± s.d. in the presence of vehicle*	*Agonist log EC50 ± s.d. in the presence of antagonist*	P-*Value (comparing agonist EC50 in the presence and absence of antagonist)*
AGN 204396 1×10^{-6} M vs prostamide F2α	−7.365 ± 0.066	−7.150 ± 0.063	0.224 NS
AGN 204396 3×10^{-6} M vs prostamide F2α	−7.065 ± 0.039	−6.713 ± 0.068	<0.0001***
AGN 204396 6×10^{-6} M vs prostamide F2α	−7. 220 ± 0.050	−6.622 ± 0.112	<0.0001***
AGN 204396 1×10^{-5} M vs prostamide F2α	−7.096 ± 0.042	−6.429 ± 0.054	<0.0001***
AGN 204396 2×10^{-5} M vs prostamide F2α	−7.237 ± 0.068	−6.210 ± 0.050	<0.0001***
AGN 204396 3×10^{-5} M vs prostamide F2α	−7.116 ± 0.059	−6.068 ± 0.055	<0.0001***
AGN 204396 3×10^{-5} M vs PGF2α	−7.780 ± 0.026	−7.780 ± 0.080	0.8722 NS
AGN 204396 1×10^{-5} M vs bimatoprost	−7.819 ± 0.057	−7.084 ± 0.037	<0.0001***
AGN 204396 3×10^{-5} M vs bimatoprost	−7.654 ± 0.052	−6.828 ± 0.065	<0.0001***
AGN 204396 1×10^{-5} M vs 17-phenyl $PGF_{2\alpha}$	−8.699 ± 0.053	−8.616 ± 0.080	0.3962 NS
AGN 204396 3×10^{-5} M vs 17-phenyl $PGF_{2\alpha}$	−8.127 ± 0.068	−8.055 ± 0.063	0.4390 NS
AGN 204396 3×10^{-5} M vs fluprostenol	−8.658 ± 0.049	−8.562 ± 0.049	0.1727 NS
AGN 204396 3×10^{-5} M vs latanoprost free acid	−7.900 ± 0.092	−7.905 ± 0.087	0.9711 NS
AGN 204396 3×10^{-5} M vs prostamide D_2	−6.259 ± 0.046	−5.302 ± 0.070	<0.001**
AGN 204396 3×10^{-5} M vs PGD_2	−7.042 ± 0.069	−6.979 ± 0.042	0.4376 NS
AGN 204396 3×10^{-5} M vs Prostamide E_2	−6.049 ± 0.049	−4.999 ± 0.060	<0.0001***
AGN 204396 3×10^{-5} M vs PGE_2	−6.500 ± 0.056	−6.126 ± 0.059	<0.0001***
AGN 204396 3×10^{-5} M vs PGE_2 - glyceryl ester	−6.155 ± 0.037	−6.174 ± 0.033	0.7161 NS
AGN 204396 3×10^{-5} M vs AL-8810	−5.167 ± 0.079	−5.079 ± 0.069	0.4175 NS
AL-8810 Ethylamide 3×10^{-5} M vs Prostamide $F_{2\alpha}$	−7.100 ± 0.072	−7.093 ± 0.047	0.9350 NS

Abbreviation: NS = non significant.
Values and log EC_{50} ± s.d.. $n = 6$ for all studies; $^{**}P < 0.01$ $^{***}P < 0.0001$.

Table 2 Antagonist activity of AGN 204396 3×10^{-5} M at human recombinant prostanoid receptor subtypes

Human prostanoid receptor	*Agonist log EC50 ± s.d. in the presence of vehicle*	*Agonist log EC50 ± s.d. in the presence of AGN 204396 3×10^{-5} M*	P *Value (comparing agonist EC50 in the presence and absence of AGN 204396 at 3×10^{-5} M)*
DP	PGD_2: −8.676 ± 0.097	PGD_2: −8.706 ± 0.091	0.8274 NS
EP_1	PGE_2: −9.074 ± 0.094	PGE_2: −9.630 ± 0.093	0.0002**
EP_2	PGE_2: −8.300 ± 0.111	PGE_2: −8.493 ± 0.110	0.2280 NS
EP_3	PGE_2: −9.403 ± 0.078	PGE_2: −9.580 ± 0.116	0.2082 NS
EP_4	PGE_2: −9.903 ± 0.065	PGE_2: −9.979 ± 0.061	0.4015 NS
FP	$PGF_{2\alpha}$: −8.442 ± 0.055	$PGF_{2\alpha}$: −8.409 ± 0.062	0.6931 NS
IP	Carbocyclin: −7.347 ± 0.077	Carbocyclin: −7.094 ± 0.078	0.0273*
TP	U-46619: −9.494 ± 0.101	U-46619: −6.175 ± 0.057	<0.0001***

Abbreviation: NS = non significant.
Values are log EC_{50} ± s.d.. $n = 3$ of experiments performed in triplicate, $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

unique, we elected to test its activity in the feline iris preparation and its susceptibility to AGN 204396 pretreatment. Although PGE_2 1-glyceryl ester produced a contraction, it was much less potent in the feline iris than in eliciting a Ca^{2+} signal in RAW 264.7 cells (Nirodi *et al*., 2004). This casts doubt on whether receptors recognizing PGE_2 1-glyceryl ester in the feline iris and RAW 264.7 cells are identical. Nevertheless, a shared identity cannot be totally excluded as, for example, $PGF_{2\alpha}$ is more than 10 times more potent as an FP receptor agonist in the rat colon compared to the mouse ileum (Woodward *et al*., 2003). What can be concluded with greater certainty is that PGE_2 1-glyceryl ester does not cause contraction of the feline iris by stimulating prostamide-sensitive receptors, as its effects are not blocked by AGN 204396.

The biological significance of a COX-2-dependent pathway for the conversion of endocannabinoids/endovanilloids to electrochemically neutral PGs remains to be elucidated. The ability of COX-2 to recognize anandamide and 2-AG may ultimately transpire to be one of its most important enzymatic functions not shared by COX-1. It has recently been demonstrated that COX-2 induction by IL-1β results in preferential and copious prostamide biosynthesis from anandamide (Glass *et al*., 2005). Moreover, prostamides show 100% crossreactivity in ELISA assays, suggesting that their presence may be widespread (Glass *et al*., 2005). The prostamides and PGE_2 1-glycerl ester appear to be pharmacologically distinct from the prostanoids (Matias *et al*., 2004; Nirodi *et al*., 2004; Chen *et al*., 2005), but at present, it is uncertain as to what their role in inflammation may be. Replacement of the charged carboxylate group of the PGs with a neutral amido or ester moiety not only alters the pharmacology but also metabolic inactivation. The stearic hindrance afforded by an ethanolamide or glyceryl ester functionality at position C1 decreases oxidation by 15-OH prostaglandin dehydrogenase (15-OH PGDH) (Kozak *et al*., 2001). This suggests that PG-ethanolamides and glyceryl esters may be longer lasting *in vivo* than PGs and may, perhaps exert their actions in tissues remote from their site of generation (Kozak *et al*., 2001). The stability of PGE_2-ethanolamide and glyceryl ester in human plasma supports this notion (Kozak *et al*., 2001). In rat plasma, PGE_2 glyceryl ester was rapidly hydrolyzed whereas prostamide E_2 was detectable in rat plasma up to 2 h after dosing, suggesting that it is significantly longer acting than PGE_2 (Kozak *et al*., 2001). The metabolic stability of endocannabinoid-derived neutral PGs opens up the possibility of additional biologically active substances with a range that extends beyond that of locally acting PGs biosynthesized from arachidonic acid. The availability of a drug, (AGN 204396), that can antagonize prostamide effects is a step forward in elucidating their biological significance.

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Conflict of interest

The authors state no conflict of interest.

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