JPET Fast Forward. Published on February 2, 2004 as DOI:10.1124/jpet.103.061705

JPET #61705

PROSTAGLANDIN-ETHANOLAMIDES (PROSTAMIDES): IN VITRO PHARMACOLOGY AND METABOLISM

I. MATIAS[#], J. CHEN[#], L. DE PETROCELLIS, T. BISOGNO, A. LIGRESTI, F. FEZZA, A.H-P. KRAUSS, L. SHI, C.E. PROTZMAN, C. LI, Y. LIANG, A.L. NIEVES, K.M. KEDZIE, R.M. BURK, V. DI MARZO[*] AND D.F. WOODWARD[*•]

Endocannabinoid Research Group, Institute of Biomolecular Chemistry (IM, TB, AL, FF, VDM) and Institute of Cybernetics (LDP), Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, 80078 Pozzuoli (Napoli), Italy; Departments of Chemical (RMB) and Biological Sciences (JC, AHK, LS, CEP, CL, YL, ALN, KMK, DFW), Allergan Inc., 2525 Dupont Drive, Irvine, CA.

JPET #61705

RUNNING TITLE PAGE

Running title: Prostamide Pharmacology and Metabolism

Author for correspondence Dr. David F. Woodward

for correspondence Dept. of Biological Sciences

Allergan, Inc.

2525 Dupont Drive (RD-2C)

Irvine, CA 92612 USA

woodward_david@allergan.com

Number of text page: 34

Number of pages: 51

Number of tables: 5

Number of figures: 10

Number of references: 39

Number of words in the *Abstract*: 235

Number of words in the *Introduction*: 733

Number of words in the *Discussion*: 1490

Abbreviations: AEA, anandamide; TRPV1, vanilloid receptor type 1; PG, prostaglandin or prostanoid; FAAH, fatty acid amide hydrolase; COX, cyclooxygenase; $CHCl_3$, chloroform; CH_3OH, methanol; TLC, thin layer chromatography; ADP, adenosine di-phosphate; ACD, acid citrate-dextrose; PRP, platelet rich plasma; EBV, Epstein Barr virus; EBNA-1, Epstein Barr nuclear antigen 1; FLIPR, fluorimetric imaging plate reader; cAMP, cyclic adenosine mono-phosphate.

Recommended section: Cellular & Molecular

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

We investigated whether prostaglandin ethanolamides (prostamides) E_2, $F_{2\alpha}$ and D_2 exert some of their effects by: i) activating prostanoid receptors either *per se* or after conversion into the corresponding prostaglandins; ii) interacting with proteins for the inactivation of the endocannabinoid *N*-arachidonoylethanolamide (AEA), thereby enhancing AEA endogenous levels; or iii) activating the vanilloid receptor type 1 (TRPV1). Prostamides potently stimulated cat iris contraction with potency approaching that of the corresponding prostaglandins. However, prostamides D_2, E_2 and $F_{2\alpha}$ exhibited no meaningful interaction with the cat recombinant FP receptor, nor with human recombinant DP, $EP_{1\text{-}4}$, FP, IP and TP prostanoid receptors. Prostamide $F_{2\alpha}$ was also very weak or inactive in a panel of bioassays specific for the various prostanoid receptors. None of the prostamides inhibited AEA enzymatic hydrolysis by fatty acid amide hydrolase (FAAH) in cell homogenates, or AEA cellular uptake in intact cells. Furthermore, less than 3% of the compounds was hydrolyzed to the corresponding prostaglandins when incubated for 4 hours with homogenates of rat brain, lung or liver, and cat iris or ciliary body. Very little temperature-dependent uptake of prostamides was observed following incubation with rat brain synaptosomes or RBL-2H3 cells. We suggest that prostamides' most prominent pharmacological actions are not due to transformation into prostaglandins, activation of prostanoid receptors, enhancement of AEA levels, or gating of TRPV1 receptors, but possibly to interaction with novel receptors that appear to be functional in the cat iris.

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

The endocannabinoid *N*-arachidonoylethanolamine (anandamide, AEA) is inactivated via a two-step mechanism, including re-uptake by cells followed by intracellular metabolism (Di Marzo, 1998). Cellular uptake occurs via facilitated transport across the plasma membrane, and is mediated by a yet-to-be characterized transporter protein (Fowler and Jacobsson, 2002). AEA hydrolysis is catalyzed by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996), whose structure, enzymatic properties, substrate selectivity and regulation have been investigated (Ueda and Yamamoto, 2000; Bisogno et al., 2002; Cravatt and Lichtman, 2002). FAAH can also efficiently catalyze the hydrolysis of other fatty acid amides, including several bioactive AEA congeners, the *N*-acylethanolamines, but also chemically different amides such as the sleep-inducing factor oleamide (Maurelli et al., 1995; Cravatt et al., 1996) and the analgesic mediator *N*-arachidonoylglycine (Burstein et al., 2002).

Evidence has accumulated pointing to the oxidation of AEA by enzymes of the arachidonate cascade, such as cytochrome p450 oxidases, lipoxygenases and cyclooxygenase-2 (COX-2) (Kozak and Marnett, 2002). In particular, COX-2, but not COX-1, recognizes AEA and catalyzes its conversion to prostaglandin endoperoxide ethanolamides, opening the way to the formation of prostaglandin E_2, D_2 and $F_{2\alpha}$ ethanolamides (Yu et al., 1997; Kozak et al., 2002), (Fig. 1), and thromboxane and prostacyclin ethanolamides (Kozak et al., 2002). Prostamides are only weakly active at cannabinoid CB_1 and CB_2 receptors (Berglund et al., 1999) and 2-3 orders of magnitude less active than the corresponding prostaglandins (PGs) in both binding and functional assays for the several prostanoid receptors known to date (Ross et al., 2002; Woodward et al., 2001; 2003). For example, prostamide E_2 is 100 to 1000-fold less potent than PGE_2 in binding assays involving human EP_1, EP_2, EP_3 and EP_4 receptor-containing membranes, about 100-fold less potent than the free acid in functional assays of EP_3 and EP_4 receptors, but

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

surprisingly only 15 times less potent than PGE_2 in EP_2 receptor mediated relaxation of the guinea pig trachea (Ross et al., 2002). The 17-phenyl-derivative of prostamide $F_{2\alpha}$, known as bimatoprost (AGN 192024, Lumigan®) (Fig. 1), exhibits very low affinity for, and potency at, the FP receptor, although it potently contracts the cat isolated iris sphincter (Woodward et al., 2001). Little is known of the pharmacology of prostamides $F_{2\alpha}$ and D_2 or of thromboxane and prostacyclin ethanolamides. The possibility that prostamides interact with another molecular target such as AEA, the vanilloid receptor type-1 (TRPV1) (Zygmunt et al., 1999; and Di Marzo et al., 2002) has been investigated only for prostamide E_2 (Ross et al., 2002).

Regarding their metabolism, it has been established that prostamides are not substrates for 15-hydroxyprostaglandin dehydrogenase, the enzyme responsible for the initial step of prostaglandin (PG) inactivation (Kozak et al., 2001), but the possibility that these compounds were substrates for FAAH or other amidases was not assessed. This is not a trivial issue since tissue-specific hydrolysis of prostamides to the corresponding prostaglandins might explain, for example, why prostamides $F_{2\alpha}$ and E_2 exhibit potency comparable to the corresponding prostaglandins in only a few assays of FP and EP_2 receptor activity (Woodward et al., 2001; Ross et al., 2002). Furthermore, if prostamides were good substrates for FAAH, they might be capable of increasing the amounts of endogenous AEA by competing for its degradation by the enzyme, as recently shown for *N*-arachidonoylglycine (Burstein et al., 2002). They would, thus, exert actions similar to those of AEA without directly interacting with the several molecular targets proposed so far for this compound (Di Marzo et al., 2002). Cellular uptake of prostamides by cells via the same mechanism responsible for AEA uptake was also assessed, since this also might lead to an enhancement of extracellular AEA levels.

In the present study we investigated whether prostamides are capable of directly activating the TRPV1 receptor, or if they serve as substrates for FAAH or the AEA membrane transporter. Furthermore, we investigated whether these compounds can be

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

hydrolyzed to the free acids in rat brain, liver or lung and in cat ciliary body or iris homogenates, or are taken up by rat brain synaptosomes or intact RBL-2H3 cells. Finally, we assessed the activity of all prostamides, in comparison with the corresponding prostaglandins, in two preparations expressing prostanoid FP receptors, as well as at cat recombinant FP receptors and human DP, $EP_{1\text{-}4}$, FP, IP and TP receptors. We report data suggesting indirectly that prostamides might owe their most potent pharmacological effects to interaction with novel and yet to be characterized molecular targets.

Materials and Methods

Materials. The 1-ethanolamides of PGD_2, PGE_2, $PGF_{2\alpha}$ and 11ß-$PGF_{2\alpha}$ were synthesized by Allergan or purchased from Cayman (Ann Arbor, MI). PGD_2, PGE_2, and $PGF_{2\alpha}$, 11ß-$PGF_{2\alpha}$ fluprostenol, carbaprostacyclin, U-46619, 17-phenyl $PGF_{2\alpha}$, and sulprostone, were purchased from Cayman (Ann Arbor, MI). $PGF_{2\alpha}$ 1-CON $(CH_3)_2$ was synthesized at Allergan. [^{3}H] PGE_2 (specific activity 165 Ci.mmol^{-1}), [^{3}H]prostamides D_2, E_2 and $F_{2\alpha}$ (specific activity 80 Ci.mmol^{-1}) and [^{3}H] 17-phenyl $PGF_{2\alpha}$ (specific activity 85 Ci.mmol^{-1}) were obtained from Amersham (Cardiff, UK). [^{3}H]-SQ 29548 (specific activity 41.5 Ci.mmol^{-1}) was purchased from NEN (Boston, MA). [^{14}C]AEA (specific activity 5 Ci.mmol^{-1}) was synthesized in our laboratory as previously described (Bisogno et al., 1997).

Intracellular calcium concentration assay. Human embryonic kidney (HEK-293) cells over-expressing the human TRPV1 receptor were a kind gift from John Davis (GlaxoSmithKline Beecham, Harlow, UK). Cells were grown as monolayers in minimum essential medium supplemented with non-essential amino acids, 10% fetal calf serum and 0.2 mM glutamine, and maintained under 95%/5% O_2/CO_2 at 37°C. The effect of substances on $[Ca^{2+}]_i$ was determined by using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+}. One day prior to experiments cells were transferred into six-well dishes coated with Poly-L-

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

lysine (Sigma) and grown in the culture medium mentioned above. On the day of the experiment the cells (50-60,000 per well) were loaded for 2h at 25°C with 4 μM Fluo-3 methylester (Molecular Probes) in DMSO containing 0.04% pluoronic. After the loading, cells were washed with Tyrode's pH=7.4, trypsinized, resuspended in Tyrode's and transferred to the cuvette of the fluorescence detector (Perkin-Elmer LS50B) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25°C (λ_{EX}=488 nm, λ_{EM}=540 nm) before and after the addition of the test compounds at various concentrations. Data are expressed as the concentration exerting a half-maximal effect (EC_{50}). The efficacy of the effect was determined by comparing it to the analogous effect observed with ionomycin (4 μM).

Cell cultures. Rat basophilic leukaemia (RBL-2H3) cells and mouse N18TG2 neuroblastoma cells were purchased from DSMZ (Germany) and grown according to the specifications of the manufacturer.

Inhibition studies of prostamides on FAAH activity. The effect of compounds on the enzymatic hydrolysis of AEA was studied as described previously (Maurelli et al., 1995), using membranes prepared from mouse neuroblastoma N18TG2 cells, incubated with the test compounds and [^{14}C]AEA (8 μM, 20,000 cpm) in 50 mM Tris-HCl, pH 9, for 30 min at 37°C. [^{14}C]Ethanolamine produced from [^{14}C]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3$/CH_3OH 2:1 (by vol.).

Inhibition studies of prostamides on AEA cellular uptake. The effect of compounds on the uptake of [^{14}C]AEA by intact rat basophilic leukemia (RBL-2H3) cells was studied by using 4 μM (10,000 cpm) of [^{14}C]AEA as described previously (Bisogno et al., 1997). Cells were incubated with [^{14}C]AEA for 5 min at 37°C, in the presence or absence of the inhibitors (50 μM). Residual [^{14}C]AEA in the incubation media after extraction with $CHCl_3$/CH_3OH 2:1

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

(by vol.), determined by scintillation counting of the lyophilized organic phase, was used as a measure of the AEA that was taken up by cells. Previous studies (Bisogno et al., 1997) had shown that, after a 5 min incubation, the amount of [^{14}C]AEA disappeared from the medium of RBL-2H3 cells is found mostly (>90%) as unmetabolized [^{14}C]AEA in the cell extract. Non-specific binding of [^{14}C]AEA to cells and plastic dishes was determined in the presence of 100 μM AEA and was never higher than 30%.

Metabolic studies with [^{3}H]-prostamides and bimatoprost. Rat brain, liver and lung, and cat ciliary body and iris, were homogenized in Tris-HCl 50 mM (pH 7.4). Debris was spun down at 800 × *g* and the protein concentration of each supernatant was determined by the Bradford method. Ten μM (20,000 cpm) of [^{3}H]prostamide D_2 or E_2 or $F_{2\alpha}$ were incubated for 4 hours at 37°C in 50 mM of Tris buffer pH 7.4 or 9.0 with 0.5-1 mg of protein. The reaction mixture was then acidified to pH 3.0 with HCl (1N) and extracted three times with ethyl acetate (2 vol.). Control incubations were carried out in the same conditions but without homogenates. A control for each experiment was also carried out using denaturated proteins (inactivated at 100°C for 10 min). The solvent was then evaporated under nitrogen and then brought up in 80 μl ethyl acetate. The samples, together with the corresponding prostamide and prostaglandin standards, were then analyzed by thin layer chromatography (TLC) carried out on silica gel G-60 layers on plastic plates which were eluted with 70:30:1 chloroform/methanol/NH_4OH for prostamide D_2 and $F_{2\alpha}$ or with 75:25:1 chloroform/ methanol/ NH_4OH for prostamide E_2. The TLC plates were then visualized by brief exposure to iodine vapors and then scanned for radioactive bands. [^{14}C]AEA (10 μM, 40000 cpm) hydrolysis to ethanolamine by brain, liver and lung homogenates was used as a positive control under the same conditions described above. After the incubation, 1 vol. of chloroform/methanol 2:1 (by vol.) was added, the mixture vortexed, and the radioactivity of

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

the aqueous phase, which contains [^{14}C]ethanolamine produced from [^{14}C]AEA hydrolysis, was measured directly by a beta-counter.

Bimatoprost (0.5 mg) was incubated with 0.5-1 mg of rat brain, cat ciliary body and iris protein (or without, for the control reaction) for 4 hours at 37°C in 50 mM of Tris buffer pH 7.4. The reaction mixture was treated and extracted under the same conditions previously described, with bimatoprost and $PGF_{2\alpha}$ as standards, on G-60 silica gel TLC glass plates, which were eluted with 95:5:1 ethyl acetate/ methanol/acetic acid and then visualized with iodine vapors.

Cellular and synaptosomal uptake studies with prostamides. For cellular uptake studies, confluent RBL-2H3 cells in six-well dishes were incubated with [^{3}H]-prostamide E_2, D_2 or $F_{2\alpha}$ (5μM, 30,000 cpm) for increasing intervals of time (0, 5, 15 and 30 min) at 37°C or 4°C. For synaptosomal uptake studies, rat brain synaptosomes were prepared as described previously by Maccarrone and coworkers (Maccarrone et al. 2001) and were incubated for different intervals of time (0, 5, 10 and 20 min) at 37°C or 4°C with prostamide E_2 or $F_{2\alpha}$ (5 μM, 30,000 cpm). Cellular and synaptosomal uptake of [^{14}C]AEA (2.5 μM, 10,000 cpm) were examined under the same conditions to verify the integrity of the transport mechanism. After the incubation, cells or synaptosomes were placed on ice and both the incubation media and cells or synaptosomes were separately extracted with chloroform/methanol (2:1, v/v) or chloroform/methanol/serum-free medium (2:1:1, v/v/v), respectively, as described previously (Bisogno et al., 1997). The organic extracts containing residual [^{3}H]prostamides from both the incubation media and cells or synaptosomes were lyophilized and radioactivity was measured by scintillation counting. Data are expressed as percent of controls and are mean ± S.E.M. of *n*=3.

Isolated Tissue Studies. Smooth muscle tension of the isolated tissues was measured isometrically with force displacement transducers (Grass FT-03) and was recorded on Grass

Polygraphs (Models 7G and 79E). The organ baths contained Krebs solution maintained at 37°C and gassed with 95% O_2/5% CO_2 to give a pH of 7.4. The Krebs solution had the following composition (mM): NaCl, 118.0; KC1, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 1.9; $MgSO_4$, 1.18; $NaHCO_3$, 25.0; glucose, 11.7; indomethacin, 0.001.

Cat Iris. Adult domestic cats were euthanized by intravenous overdose of sodium pentobarbital (Anthony, Arcadia, CA). The eyes were enucleated immediately and placed on ice. The iris sphincter muscle was mounted vertically under 50 to 100 mg tension in a jacketed 10 ml organ bath. A 60 min stabilization period was allowed before commencing each experiment. Activity was determined as contractile responses. Compounds were added cumulatively to the organ bath and at least 30 min was allowed for recovery, after complete wash-out and return to baseline tension. The response to 10^{-7} M $PGF_{2\alpha}$ was determined at the beginning and end of each experiment and between dose-response curves as a reference.

Endothelium-intact rabbit jugular vein. New Zealand albino rabbit of either sex, weighing 2-4 kg, were injected with 1000 U heparin into the marginal ear vein and then euthanized by CO_2 gas inhalation. The external jugular veins were cleared of fat and adherent connective tissue and surgically excised. The veins were transected and each ring of 3-4 mm length was suspended between two metal hooks in a jacketed organ bath. The tissues were equilibrated for 1 hr under 0.75 g tension, which was readjusted as the tissues relaxed. Single doses of histamine, 10^{-5} M then 2-3 x 10^{-6} M, with washing after each dose, were given to contract the tissue and establish responsiveness. The TP-receptor antagonist SQ29548, (10^{-6} M), was applied for 5 min and then histamine 2-3 x 10^{-6} M was added to elicit the contractile response. After 30 min of pretreatment with histamine, the relaxant response was tested by adding cumulative doses of the test compounds, with 10^{-8} M to 10^{-7} M PGE_2 at the end of each dose-response curve as a reference. A recovery period of 30-50 min was allowed after

wash-out of the tissues. Relaxant activity was calculated as % of the control tone elicited by histamine.

Guinea Pig Ileum. Guinea pig (Hartley) ileum preparations of approximately 1.5 cm in length were suspended under 1.0 g tension. After a 1 hr equilibration period, a standard dose-response to PGE_2 was obtained in a non-cumulative manner with 30 min washout periods between individual doses. Subsequently, graded doses of test compounds were added non-cumulatively. A maximal dose of PGE_2 (10^{-6} M) was given as the terminal step of the experiment and served as a second reference response. Contractile activity at each concentration was then calculated as % of the 10^{-6} M PGE_2 response.

Guinea Pig Vas Deferens. A 1.5 cm portion of guinea pig vas deferens was suspended longitudinally under the 1.0 g tension and allowed to equilibrate for at least 30 min without electrical stimulation. Tissues were then subjected to stimulation every 30 s by a train of electrical impulses. Each train consisted of 10 x 20V pulses of 1 ms duration, each individual pulse separated by 100 ms. Electrical current was generated by a Grass S48 stimulator (Grass Instruments) and distributed to the individual organ baths with a Stimu-splitter II (Med-Lab Instruments, Loveland, CO, USA), using glass tissue supports with stimulating platinum electrodes in parallel orientation to the tissues (Radnoti Glass Technology, Monrovia, CA, USA). After stabilization of the twitch response, PGE_2 was applied cumulatively. Investigational compounds were then evaluated in a cumulative manner. PGE_2 was re-applied at the conclusion of the experiment as a reference standard. One hour was allowed to elapse between testing of PGE_2 and the investigational compound. Activity was calculated as % inhibition of the muscle twitch response.

Rat Aorta. Adult rats of the Sprague-Dawley strain were used. The thoracic aorta was surgically excised and cleaned of any adhering tissue. Tissue segments of 5-8 mm length were prepared. Each segment was mounted under 2.0 g tension. The tissue preparations were

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

allowed to equilibrate for 1 hr before compounds were tested in a cumulative manner. The response to 100 nM U-46619 was determined at the beginning and end of each experiment as a reference standard. A 30-45 min was allowed for tissue recovery after washout of each drug.

Cell studies.

(a) *Human platelets*. Activity at DP-, TP- and IP-receptor subtypes was determined by an ability to cause aggregation (TP-receptor activity) or to inhibit ADP-induced aggregation of human platelets *in vitro* (DP- and IP-receptor activity). Fresh whole blood was obtained from consenting healthy human volunteers and mixed with acid citrate-dextrose (ACD). The blood was centrifuged at 1000 rpm for 15-20 minutes to obtain platelet rich plasma (PRP). 4.5 µl of prostanoid solution or vehicle was added to 450 µl of PRP and incubated for 2 minutes at 37° C in a Payton aggregometer and observed for any aggregatory activity. 2×10^{-5} M ADP (final concentration) was then added to induce full aggregation. Inhibition of aggregation was calculated as the % difference between aggregation evoked by 2×10^{-5} M ADP in the absence and presence of drug. Aggregatory activity was calculated as the % aggregation in response to the prostamide or prostanoid relative to the aggregation induced by 2×10^{-5} M ADP. Standard aggregatory responses to 2×10^{-5} M ADP alone were performed at the beginning and end of each experiment.

(b) *Human recombinant EP_1, EP_2, EP_4, FP and TP receptors: binding studies.* Plasmids encoding the human EP_1, EP_2, EP_4, and FP receptors were prepared by cloning the respective coding sequences into the eukaryotic expression vector pCEP4 (Invitrogen; Carlsbad, CA). The pCEP4 vector contains an Epstein Barr virus (EBV) origin of replication, which permits episomal replication in primate cell lines expressing EBV nuclear antigen (EBNA-1). It also contains a hygromycin resistance gene that is used for eukaryotic selection. The cells employed for stable transfection were human embryonic kidney cells (HEK-293) that were

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

transfected with and express the EBNA-1 protein. These HEK-293-EBNA cells (Invitrogen) were grown in medium containing geneticin (G418) to maintain expression of the EBNA-1 protein. HEK-293 cells were grown in DMEM with 10% fetal bovine serum (FBS), 250 μg ml^{-1} G418 (Life Technologies; Hercules, CA) and 200 μg ml^{-1} gentamicin or penicillin/streptomycin. Selection of stable transfectants was achieved with 200μg ml^{-1} hygromycin, the optimal concentration being determined by previous hygromycin kill curve studies. For prostanoid receptor transfection, the cells were seeded into 6 well plates at a density of 10^5/well the day before transfection. Fugene 6 transfection agent (Roche; Indianapolis, IN) was diluted in OPTI-MEM (Gibco-BRL). The pCEP4 expression vector, containing cDNA for the required prostanoid receptor was then added and incubated for 15 min at room temperature. The mixture was added such that each well received 3 μl of Fugene 6 solution and 1 μg $pCEP_4$ vector-prostanoid receptor cDNA. This was followed by two days incubation. The cells were then transferred into 5 x 100 mm dishes with the cells attached. The medium was replaced by selection medium containing 200 μg ml^{-1} hygromycin B. Hygromycin B resistant clones were individually selected and transferred to separate 24 well plates. At confluence, each clone was transferred to one well of a 6 well plate and then expanded. Cells were maintained under continuous hygromycin selection as above until use.

(c) *Human recombinant EP_3 and TP receptors: binding studies.* Plasmids encoding the human EP_{3D} isoform (Regan et al., 1994) or TP receptor were prepared by cloning the respective coding sequences into pcDNA3 vector (Invitrogen). COS-7 cells were transfected with pcDNA3 containing cDNA encoding the EP_3 or TP receptor by employing the lipofectin method, according to the manufacturers instructions (Gibco-BRL). For radioligand binding studies, cells were harvested two days after transfection.

(d) *Radioligand binding*. Radioligand binding studies using plasma membrane fractions prepared from cells stably expressing the cat or human receptor were performed as follows.

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

Cells were washed with TME buffer, scraped from the bottom of the flasks, and homogenized for 30 s using a Brinkman PT 10/35 polytron. TME buffer was added as necessary to achieve a 40 ml volume in the centrifuge tubes. TME is composed of 50 mM TRIS base, 10 mM $MgCl_2$, 1 mM EDTA; pH 7.4 was achieved by adding 1N HCl. The cell homogenate was centrifuged at 19,000 rpm for 20-25 min at 4°C using a Beckman Ti-60 or Ti-70 rotor. The pellet was then resuspended in TME buffer to provide a final protein concentration of 1 mg ml^{-1}, as determined by Bio-Rad assay. Radioligand binding assays were performed in a 100 µl or 200 µl volume. Binding was determined in duplicate for at least 3 separate experiments. Incubations were for 60 min at 25° C and were terminated by the addition of 4 ml of ice-cold 50 mM TRIS-HC1, followed by rapid filtration through Whatman GF/B filters and three additional 4 ml washes in a cell harvester (Brandel). Competition studies were performed using a final concentration of 2.5 or 5 nM [^{3}H]-PGE_2 (EP_1, EP_2 and EP_4 receptors) or 5 nM [^{3}H]-17-phenyl $PGF_{2\alpha}$ (FP receptors). Non-specific binding was determined with 10^{-5} M of unlabeled ligand. For radioligand binding on the transient transfectants, plasma membrane fraction preparation was as follows. COS-7 cells were washed with TME buffer, scraped from the bottom of the flasks, and homogenized for 30 sec using a Brinkman PT 10/35 polytron. TME buffer was added to achieve a final 40 ml volume in the centrifuge tubes. The cell homogenate was centrifuged at 19,000 rpm for 20 min at 4°C using a Beckman Ti-60 rotor. The resultant pellet was resuspended in TME buffer to give a final 1 mg/ml protein concentration, as determined by Bio-Rad assay. Radioligand binding assays were performed in a 100 µl or 200 µl volume. Competition binding vs [^{3}H]-PGE_2 at EP_{3D}, receptors or [^{3}H]-SQ29548 at TP receptors was determined in duplicate in at least three separate experiments. Incubations were for 60 min at 25°C and were terminated by the addition of 4 ml of ice-cold 50 mM TRIS-HCl, followed by rapid filtration through Whatman GF/B filters and three additional 4 ml washes in a cell harvester (Brandel). Competition studies were performed

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

using a final concentration of 5 nM [^{3}H]-PGE_2, or 10 nM [^{3}H]-SQ 29548 and non-specific binding was determined with 10^{-5} M of the respective unlabeled prostanoid.

(e) *Human DP Receptor Luciferase Reporter Assay for hDP-HEK 293/EBNA.* Stable DP receptor transfectants were prepared as previously described (b) for EP_1, EP_2, EP_4, FP and TP receptors. For the luciferase reporter assay, 5 x 10^4 hDP-HEK 293/EBNA cells were seeded in a 24 well plate the day before transfection. The CRE-luciferase reporter plasmid (Stratagene, La Jolla, CA) was transiently transfected into the above cells by the Fugene 6 method. Compounds at a concentration range from 10^{-11} to 10^{-6} M were added to the culture for 6 hr after overnight transfection. The cells were harvested and lysed in 100 µl of 25 mM Tris-phosphate buffer (pH 7.5) containing 1% Triton X-100. 20 µl of soluble extracts were assayed for luciferase. The luciferase assay was performed with a Promega assay kit (Promega; Madison, WI). Light intensity was measured by Lumat. Relative luciferase activity was expressed as ratio compared to control.

(f) Human IP Receptor Luciferase Reporter Assay for hIP-HEK 293/ EBNA. Stable IP receptor transfectants were prepared as previously described (b). Cells for the luciferase reporter assay were prepared as follows. hDP-HEK 293/EBNA cells were seeded in a 24 well plate 24 hr before transfection. The CRE-luciferase reporter plasmid (Stratagene, La Jolla, CA) was transiently transfected into the above cells by the Fugene 6 method. Compounds at a concentration range from 10^{-11} to 10^{-6} M were added to the culture for 6 hr after overnight transfection. The cells were harvested and lysed in 100 µl of 25 mM Tris-phosphate buffer (pH 7.5) containing 1% Triton X-100. Soluble extracts (50 µl) were assayed for luciferase. The luciferase assay was performed with a Promega assay kit. Light intensity was measured by Lumat. Relative luciferase activity was expressed as ratio compared to control.

(g) *Human recombinant DP, EP_2, EP_3 and EP_4 receptors: Ca^{2+} Signaling (FLIPR) Studies.* The use of chimeric G protein cDNAs allowed responses to Gs and Gi coupled

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

receptors to be measured as a Ca^{2+} signal. Prostanoid DP, EP_2, and EP_4 receptor cDNAs were co-transfected with chimeric Gqs cDNA containing an HA epitope. The prostanoid EP_3 receptor was contransfected with chimeric Gqi-HA. Gqs^5 and Gq_i^5 chimeric cDNAs (Molecular Devices, Sunnyvale, CA) were also cloned into $pCEP_4$ vector and were selected by means of a hygromycin B selection marker. Transfection into HEK-EBNA cells was accomplished by the Fugene-6 method as previously described. Since the Gqs and Gq chimerics contain an HA epitope, protein expression may be detected by a specific antibody. In Western blot experiments, anti-HA-peroxidase and a mouse monoclonal antibody (clone 12 CA5) were employed.

(h) *Human recombinant EP_1, IP, FP and TP receptors: FLIPR studies*. Stable transfectants were obtained as described for radioligand binding studies (b). Briefly, $pCEP_4$ was used as the expression vector and transfection into HEK-293-EBNA cells was achieved using Fugene 6. Stable transfectants were selected according to hygromycin resistance.

(i) *Ca^{2+} signal studies using fluorometric imaging plate reader (FLIPR™).* Cells were seeded at a density of $5x10^4$ cells per well in Biocoat® Poly-D-lysine-coated black-wall, clear-bottom 96-well plates (Becton-Dickinson) and allowed to attach overnight in an incubator at 37°C. Cells were then washed twice with HBSS-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). After 45-60 min of dye-loading in the dark using the calcium-sensitive dye Fluo-4 AM, at a final concentration of 2 µM, plates were washed four times with HBSS-HEPES buffer to remove excess dye and leaving 100µl buffer in each well. Plates were then placed into a FLIPR™ instrument and were allowed to equilibrate at 37°C. Drug solution was 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). The peak increase in fluorescence intensity was recorded for each well. On each plate, four wells each served as negative (HBSS-HEPES buffer) and positive controls (standard agonist, depending on receptor). The peak fluorescence change in each drug-containing well was then expressed relative to the controls. To generate concentration-response curves, compounds were tested in duplicate in each plate over the desired concentration range. The duplicate values were averaged. Each compound was tested on at least 3 separate plates using cells from different passages to give an $n = 3$.

Statistical analyses. All data are presented as mean ± S.E.M. Statistical analysis was performed using analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's test. A P value of 0.05 or less was considered significant.

Results

Effects of prostamides on cat iris sphincter contraction. The effects of PGD_2, PGE_2, $PGF_{2\alpha}$ and 11ß-$PGF_{2\alpha}$ were compared to the activities of the corresponding ethanolamide analogs (whose chemical structures are shown in Fig. 1) in the cat isolated iris sphincter preparation. These data are depicted in Fig. 2. Prostamide $F_{2\alpha}$ was the most potent prostamide, with an EC_{50} value of 57 nM. Prostamides D_2 and E_2 were essentially equiactive but were approximately 10-fold less active then prostamide $F_{2\alpha}$, with EC_{50} values of 499 and 564 nM, respectively (Fig. 2). The corresponding free acids of prostamides D_2, E_2, and $F_{2\alpha}$ were only marginally more active than the prostamides: EC_{50} (nM) values were $PGF_{2\alpha}$ = 11, PGD_2 = 150, PGE_2 = 260. A marked separation between the activity of 11ß-$PGF_{2\alpha}$ and the corresponding ethanolamide was apparent: EC_{50} values were 11ß-$PGF_{2\alpha}$ = 54, 11ß-prostamide = 1827. The activity of $PGF_{2\alpha}$ 1-CON $(CH_3)_2$ was also evaluated in the cat iris and an EC_{50} value of 450 nM was obtained (data not shown).

Effect of prostamides on the cat recombinant FP receptor. Since the cat iris sphincter is a tissue preparation with particular sensitivity to prostamides, activity at the cat recombinant FP receptor was determined to investigate whether prostamide activity was a species related phenomenon. This did not appear to be the case. Prostamide interaction at the feline FP receptor was no more than residual compared to $PGF_{2\alpha}$ activity (Fig. 3). This was confirmed for prostamides D_2, E_2 and $F_{2\alpha}$ by radioligand binding (Fig. 4). The activity of the natural PGs at cat recombinant FP receptors showed the typical potency rank order $PGF_{2\alpha}$ > PGD_2 > PGE_2 that has been previously described for other preparations (Coleman et al., 1984). EC_{50} values [nM] at the cat recombinant FP receptor were $PGF_{2\alpha}$ = 6.8, 11ß-$PGF_{2\alpha}$ = 37; PGD_2 = 40, PGE_2 = 396; all prostamides > 10,000. $PGF_{2\alpha}$ 1-CON (CH_3) exhibited no meaningful activity at the cat recombinant FP receptor (EC_{50} >10,000 data not shown).

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

Effect of prostamides on the pre-contracted rabbit jugular vein. The relaxant effects of prostamides D_2, and $F_{2\alpha}$ on the pre-contracted rabbit jugular vein are compared to those of PGD_2, and $PGF_{2\alpha}$ in Fig. 5. In marked contrast to the cat iris sphincter (Fig. 2a), a substantial difference between the potency of $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ was apparent in the pre-contracted rabbit jugular vein preparation. Similarly, PGD_2 was approximately two orders of magnitude more potent than prostamide D_2 (Fig. 5a). EC_{50} values (nM) were obtained as follows: $PGF_{2\alpha}$ = 2.8, prostamide $F_{2\alpha}$ = 2000; PGD_2 = 28, prostamide D_2 = 3060. The difference in potency between prostamide D_2 and PGD_2 is similar to the difference between PGE_2 and prostamide E_2 previously reported by Ross et al., (2002).

Effect of prostamides on recombinant and native prostanoid receptors. The pharmacology of prostamides was further compared to the natural PGs in functional and radioligand binding competition studies utilizing human recombinant prostanoid receptors. Ca^{2+} signaling studies at all major receptor subtypes were performed using a FLIPR™ instrument. These Ca^{2+} signaling data are summarized in Table 1 and compared to relative activities in the cat iris preparation. Prostamides D_2 and $F_{2\alpha}$ were essentially inactive in terms of stimulating human recombinant receptors. Prostamide E_2 was more active than prostamides D_2 and $F_{2\alpha}$. A measurable EC_{50} value was obtained for prostamide E_2 at the EP_1 receptor (848 nM) and the EP_3 receptor (123 nM); PGE_2 had potent activities at EP receptors, however (subnanomolar at $EP_{1,3,4}$ and 2.5 nM at EP_2). Prostamide E_2 activity was negligible at EP_2 and EP_4 receptors and no effect was apparent until a 10^{-5} M concentration was achieved. The compound was inactive at DP, FP, IP and TP receptors.

Radioligand competition binding studies were performed at all prostanoid receptor subtypes, with the exception of DP and IP receptors due to the lack of availability of high affinity, high specific activity radioligands. Prostamide D_2 exhibited no apparent affinity for $EP_{1\text{-}4}$, FP, or TP receptors. Prostamide E_2 exhibited weak affinity for the EP_4 receptors.

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

Prostamide $F_{2\alpha}$ exhibited weak affinity for the FP receptor and the $EP_{2\text{-}4}$ and TP receptors. These data are summarized in Table 2.

In addition to studies on DP and IP receptors using the FLIPR™ Ca^{2+} signaling technique, a luciferase reporter assay was used to indirectly measure cAMP mediated effects. No effect was apparent up to a 10^{-6} M concentration for prostamides D_2, E_2 and $F_{2\alpha}$. PGD_2 and carbaprostacyclin served as positive controls and behaved as potent agonists at DP and IP receptors, with respective EC_{50} values of 40 and 5 nM (data not shown).

The activity of prostamide $F_{2\alpha}$ was also compared functionally in a range of cell and isolated tissue preparations. The data obtained are given in Table 3. Prostamide $F_{2\alpha}$ was essentially inactive at human DP, IP and TP receptors associated with platelets. In the isolated tissue and cell preparations prostamide $F_{2\alpha}$ exhibited modest activity and EC_{50} values exceeded 10^{-6} M. Prostamide $F_{2\alpha}$ was much less potent than the respective selective prostanoid receptor agonist for each preparation: guinea pig ileum (EP_1), guinea pig vas deferens (EP_3), endothelium-intact rabbit jugular vein (FP), and rat aorta (TP).

Effect of prostamides on TRPV1 receptors. The possible functional interaction of prostamides with TRPV1 receptors was studied by using an intracellular calcium concentration assay in intact HEK-293 cells over-expressing the human TRPV1 receptor. Previous studies have shown that this assay is more sensitive than other TRPV1 assays since, for example, the EC_{50} values for AEA and capsaicin in this assay are usually at least one order of magnitude higher than those observed with ion current measurements and up to 100-fold higher than the corresponding K_i values obtained using binding assays. This is presumably because the initial calcium influx triggered by TRPV1 gating is amplified by intracellular calcium mobilization. Using this assay we found that, of the three prostamides, only prostamide $F_{2\alpha}$ was capable of enhancing intracellular calcium with an $EC_{50} = 15.0 \pm 2.4$ μM and a maximal effect that was 31 ± 2% of the maximal effect observable with 4 μM of

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

ionomycin (mean ± SEM, *n* = 3) (Fig. 6). Prostamide D_2 also exerted a measurable effect but with a significantly lower efficacy, whereas, in agreement with previous data obtained by using a binding assay (Ross et al., 2002), prostamide E_2 was almost inactive in this test (Fig. 6). Bimatoprost did not elicit any response up to a 25 μM concentration (data not shown). Under the same conditions AEA and capsaicin exhibit EC_{50} values of 540 ± 120 and 35 ± 8 nM, respectively. The effect of prostamide $F_{2\alpha}$ (50 μM) was blocked by the TRPV1 antagonist, capsazepine (1 μM, from 31 ± 2 to 5 ± 2% of the maximal effect of ionomycin, mean ± SEM, *n* = 3, $P<0.05$); no effect was observed in non-transfected HEK-293 cells (data not shown). Since, in order to activate TRPV1, many agonists, including AEA, need to interact with an intracellular binding site (Jung et al., 1999; De Petrocellis et al., 2001), and since prostamide $F_{2\alpha}$ appeared to be only slowly transported into cells (see above), we incubated HEK cells with this compound in the presence of pluronic, which increases the permeability of the cell membrane to some extent. This treatment, while slightly enhancing the maximal effect of prostamide E_2 and $F_{2\alpha}$, did not affect prostamide D_2 action on intracellular calcium (Fig. 6).

Effect of prostamides on FAAH activity. The effect of prostamides on FAAH was studied using membrane preparations from mouse N18TG2 cells, which contain high levels of the enzyme (Maurelli et al., 1995). None of prostamides, nor bimatoprost, exerted any significant inhibition of [^{14}C]AEA hydrolysis by N18TG2 cell membranes at any of the concentrations tested and up to 100 μM (Fig. 7).

Effect of prostamides on AEA cellular uptake. The effect of prostamides on AEA cellular uptake was studied in intact RBL-2H3 cells, where the putative AEA membrane transporter has been thoroughly studied (Bisogno et al., 1997). None of the three prostamides, nor bimatoprost, exerted any significant inhibition of [^{14}C]AEA uptake by RBL-2H3 cells at any of the concentrations tested and up to 50 μM (Fig. 8).

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

Metabolic stability of prostamides in homogenates from various tissues. The enzymatic hydrolysis of the prostamides was assessed by using the corresponding [^{3}H]-labelled compounds. In whole rat brain, lung or liver homogenates, or with cat ciliary body and iris homogenates, incubated with the compounds at pH optimal either for FAAH (9.0) or for other hydrolases (7.4), only very small amounts, if any, of [^{3}H]-labelled corresponding prostaglandins were formed (Tables 4, 5). Under the same conditions, 50-100% of [^{14}C]AEA was hydrolyzed to [^{14}C]ethanolamine (Tables 4, 5). Likewise, no hydrolysis product (i.e. 17-phenyl-$PGF_{2\alpha}$) was found after incubation of bimatoprost with homogenates of rat brain (data not shown), cat ciliary body (data not shown) and cat iris (Fig. 9).

Cellular and synaptosomal uptake of prostamides. The cellular uptake of prostamides was assessed again by using the corresponding [^{3}H]-labelled compounds. When the compounds were incubated with intact RBL-2H3 cells, or with rat brain synaptosomes, only negligible amounts were taken up from the incubation medium in a temperature-dependent way and after up to 30 min incubations (Fig. 10A,B). However, unlike RBL-2H3 cells, the very modest uptake by synaptosomes was statistically significant ($P<0.05$). Under the same conditions, [^{14}C]AEA was taken up by the cells much more efficaciously (Fig. 10A,B).

Discussion

We present evidence that: 1) prostamides E_2, D_2 and $F_{2\alpha}$ have very little, if any, affinity for prostanoid receptors; 2) prostamide $F_{2\alpha}$ potently contracts the feline iris sphincter independent from FP receptors; 3) prostamide $F_{2\alpha}$, and much less so prostamides E_2 and D_2, directly activate the TRPV1 receptor, albeit at concentrations higher than 10 μM and with low efficacy compared to capsaicin, or AEA; 4) prostamides E_2, D_2 and $F_{2\alpha}$ have very little, if any, affinity for, and do not inhibit the activity of the two proteins mostly responsible for AEA inactivation; this indicates that prostamides, unlike other bioactive amides such as N-arachidonoylglycine (Burstein et al., 2002) are not likely to act by elevating levels of AEA and resultant activation of AEA molecular targets; 5) prostamides are very stable to hydrolysis by enzymatically active rat brain, liver, lung and cat ciliary body and iris homogenates; 6) there is no efficient uptake by intact RBL-2H3 cells or rat brain synaptosomes. These observations, together with the poor affinity of prostamides for cannabinoid CB_1 and CB_2 receptors (Berglund et al., 1999), support the hypothesis that prostamides E_2 and $F_{2\alpha}$ might by interact with novel receptors. Furthermore, our data suggest that COX-2-catalyzed oxidation of AEA to prostamides represents an inactivation step with respect to the AEA, not only at cannabinoid receptors but also at vanilloid TRPV1 receptors.

Previous prostaglandin-ethanolamides studies were limited to comparison of either prostamide E_2 (Ross et al., 2002) or prostamide $F_{2\alpha}$ (Woodward et al., 2001) with their corresponding prostaglandins. In this present study we directly compared the activity of four different prostamides in an isolated tissue preparation that is sensitive to prostamide $F_{2\alpha}$, namely the cat iris sphincter. The results were unexpected. The potencies of prostamides D_2, E_2, and $F_{2\alpha}$ did not differ markedly. This did not correlate with their very weak affinity and functional potency in systems involving recombinant and natural prostanoid receptors. The

feline iris sphincter appears distinctly sensitive to prostamides D_2, E_2 and $F_{2\alpha}$, suggesting the possible existence of receptors that preferentially recognize these molecules.

The sensitivity of the cat iris sphincter to prostamides is not a phenomenon generally associated with isolated smooth muscle preparations. A series of $PGF_{2\alpha}$-amides was studied in the gerbil isolated colon (Maddox et al., 1978), which is an FP receptor preparation (Coleman et al., 1984). Comparing the activity of a limited series of 1-amido $PGF_{2\alpha}$ analogs with $PGF_{2\alpha}$, replacement of the –COOH group of $PGF_{2\alpha}$ with a $–CONH_2$ moiety produced about a 300 fold reduction in potency. The monomethyl and dimethyl amide analogs of $PGF_{2\alpha}$ were even less active (Maddox et al., 1978). Despite their lack of activity in the gerbil colon, this and a previous study (Woodward et al., 2001) have shown that $PGF_{2\alpha}$ analogs containing an amido moiety are essentially equipotent with $PGF_{2\alpha}$ in contracting the cat iris sphincter. Antagonist properties have been claimed for $PGF_{2\alpha}$ 1-CON $(CH_3)_2$ (Maddox et al., 1978) but in the feline iris sphincter this compound behaved as a full agonist. These early studies on the antagonist properties of $PGF_{2\alpha}$ 1-dimethylamide are arguably deficient since the $PGF_{2\alpha}$ dose-range employed was too narrow (Maddox et al., 1978). A recent study claimed that $PGF_{2\alpha}$ 1-dimethylamide is not an FP receptor antagonist (Sharif et al., 2001). It is, however, interesting to note that in both antagonist studies no meaningful stimulation of the FP receptor was reported (Maddox et al., 1978; Griffin et al., 1999). This is in contrast to these present studies on the cat iris, where clear agonist activity was apparent.

The activity of the prostamides was also studied in an isolated tissue preparation that contains FP receptors and PGD_2 and PGE_2-sensitive receptors, namely the rabbit isolated jugular vein. It contains FP receptors associated with vascular endothelial cells (Chen et al., 1995; Chen and Woodward, 2002), and DP, EP_2 and EP_4 receptors associated with vascular smooth muscle (Giles et al., 1989; Nials et al., 1991; Lawrence and Jones, 1992; Milne et al., 1995). The results obtained in the rabbit jugular vein were quite different from those obtained

in the cat iris sphincter. In the jugular vein, prostamides were more than two orders of magnitude less potent than the corresponding prostaglandins. These results are essentially consistent with a previous report, where PGE_2 was found to be about 200 times more potent than prostamide E_2 in the rabbit jugular vein (Ross et al., 2002).

Prostamides D_2, E_2 and $F_{2\alpha}$ were also compared to the activity of the corresponding PGs in functional assays and radioligand binding competition studies involving human recombinant receptors. In every instance, an ethanolamide substituent at position-1 resulted in a dramatic reduction in functional potency and affinity for PG receptors. The cat FP receptor was also studied to determine if species differences could account for the high potency of the prostamides in the cat iris sphincter preparation. This was not the case. Thus, only in the cat iris are PGD_2, PGE_2 and $PGF_{2\alpha}$ approximately equipotent to the corresponding prostamides.

We investigated whether prostamides could interact with vanilloid TRPV1 receptors because this protein is efficaciously activated by a prostamide putative biosynthetic precursor, AEA (Zygmunt et al., 1999). Furthermore, stimulation of TRPV1 receptors results in vasodilatation with a tendency to de-sensitize (Zygmunt et al., 1999). These events are characteristic of bimatoprost-induced ocular surface hyperemia (Abelson et al., 2003). However, interaction with TRPV1 is not likely to underlie this effect of bimatoprost, nor the other pharmacological actions that this compound shares with prostamide $F_{2\alpha}$, such as potent contraction of the cat iris sphincter (Woodward et al., 2001), since only prostamide $F_{2\alpha}$ was found here to exert an appreciable, albeit very weak, TRPV1-mediated effect.

Apart from the possible existence of a specific receptor for prostamides, the high potency of these compounds in the cat iris could also be explained by their rapid hydrolysis to the corresponding prostaglandins. However, the present experiments seem to exclude this possibility since, with all tissues used here, including the cat iris sphincter where the

compounds are particularly potent, prostamides were extremely stable to enzymatic hydrolysis. The lack of appreciable enzymatic hydrolysis of prostamides by FAAH after a physiologically relevant period of time (≤4 hours) is in agreement with the structural requirements of this enzyme, which recognizes more efficiently as substrates fatty acid amides with little or no derivatization of the fatty acid chain, and a preferential "J" conformation in solution (Reggio, 2002). Furthermore, our finding of little enzymatic hydrolysis in rat brain homogenates, where non-FAAH hydrolases are likely be present, agrees with a previous study showing that prostamide E_2 is not hydrolyzed in either rat or human plasma (Kozak et al., 2001). Taken together with previous observations, our data on the refractoriness of prostamides to both enzymatic hydrolysis and cellular re-uptake support the notion that these compounds are metabolically stable, inactivation products of AEA.

We also found that the prostamide analogue bimatoprost (Woodward et al., 2001; 2003), did not compete with AEA for FAAH or the AEA membrane transporter, suggesting that this compound is not a substrate for these two proteins. Bimatoprost was also unmetabolized after a 4 hour incubation with homogenates of rat brain, lung and liver, or of cat iris and ciliary body. These findings are in agreement with the high ocular metabolic stability, reported in vivo (Woodward et al., 2003) and in vitro (Maxey et al., 2002), where conversion to the corresponding free acid to a barely detectable extent occurs only after very long (≥6 hours) incubations (Maxey et al., 2002) The absence of prostamide $F_{2\alpha}$ conversion to a free acid metabolite is also consistent with data obtained with the ester latanoprost in the cat isolated iris: here latanoprost was referred to as pharmacologically inactive (Resul et al., 1997) or only weakly active (Woodward et al., 2001).

In conclusion, we have provided data allowing us to rule out that prostamides and bimatoprost are metabolized to the free acids or act by inhibiting the inactivation of their biosynthetic precursor AEA. These findings argue against the possibility that prostamides act

in vivo by exerting AEA-like effects via the enhancement of endogenous AEA levels. Furthermore, it is also possible to rule out that AEA oxidation by COX-2 leads to feed-back mechanisms that inhibit FAAH-catalyzed AEA inactivation, as has been suggested for AEA oxidation by lipoxygenases to products with FAAH inhibitory activity (van der Stelt et al., 2002). Our finding of weak activity by prostamide $F_{2\alpha}$ at TRPV1 receptors suggests that this receptor is not responsible for the potent pharmacological actions described for this compound (Woodward et al., 2001). Finally, the lack of high potency of the prostamides at any of the known prostanoid receptors, together with their high metabolic stability and their high potency in the cat iris preparation, provide evidence consistent with the existence of prostamide sensitive receptors in this tissue. The nature of prostamide sensitive receptors may represent a population that interacts exclusively with prostamides and other non-acid congeners such as PG-glyceryl esters (Kozak et al., 2001) and $PGF_{2\alpha}$ 1-alcohol and $PGF_{2\alpha}$ 1-methoxy (Woodward et al., 2000).

Acknowledgements

The authors express their great appreciation to Linda L. Johnson for preparation of the manuscript.

References

Abelson MB, Mroz M, Rosner SA, Dirks MS and Hirabayashi D (2003) Multicenter, open-label evaluation of hyperemia associated with use of bimatoprost in adults with open-angle glaucoma or ocular hypertension. *Adv Ther* **20**:1-13.

Berglund BA, Boring DL and Howlett AC (1999) Investigation Of Structural Analogs of prostaglandin amides for binding to and activation of CB1 and CB2 cannabinoid receptors in rat brain and human tonsils. *Adv Exp Med Biol* **469:**527-533.

Bisogno T, Maurelli S, Melck D, De Petrocellis L and Di Marzo V (1997) Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. *J Biol Chem* **272:**3315-3323.

Bisogno T, De Petrocellis L and Di Marzo V (2002) Fatty Acid Amide Hydrolase, an enzyme with many bioactive substrates. Possible therapeutic implications. *Curr Pharm Des*. **8:**533-547.

Burstein SH, Huang SM, Petros TJ, Rossetti RG, Walker JM and Zurier RB (2002) Regulation Of Anandamide Tissue Levels By N-Arachidonylglycine. *Biochem Pharmacol* **64:**1147-1150.

Chen J, Champa-Rodriguez ML and Woodward DF (1995) Identification of a prostanoid FP receptor population producing endothelium-dependent vasorelaxation in the rabbit jugular vein. *Br J Pharmacol* **116:**3035-3041.

Chen J and Woodward DF (2002) Prostanoid FP receptor-mediated, endothelium-dependent vasodilatation and the ocular surface hyperemic response to $PGF_{2\alpha}$ and related compounds, in *Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury, 5* (Honn, KV, Marnett LJ, Nigam S, Serhan CN and Dennis EA eds) pp 331-336, Kluwer Academic/Plenum Publishers, New York.

Coleman RA, Humphrey PPA, Kennedy I and Lumley P (1984) Prostanoid receptors: the development of a working classification. *Trends Pharmacol Sci* **5:**303-306.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA and Gilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384:**83-87.

Cravatt BF and Lichtman AH (2002) The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chem Phys Lipids* **121:**135-48.

De Petrocellis L, Bisogno T, Maccarrone M, Davis JB, Finazzi-Agro A and Di Marzo V (2001) The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. *J Biol Chem* **276:**12856-12863.

Di Marzo V (1998) 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance. *Biochim Biophys Acta* **1392:**153-175.

Di Marzo V, De Petrocellis L, Fezza F, Ligresti A and Bisogno T (2002) Anandamide receptors. *Prostaglandins Leukot Essent Fatty Acids* **66:**377-391.

Fowler CJ and Jacobsson SO (2002) Cellular transport of anandamide, 2-arachidonoylglycerol and palmitoylethanolamide--targets for drug development? *Prostaglandins Leukot Essent Fatty Acids* **66:**193-200.

Giles H, Leff P, Bolofo ML, Kelly MG and Robertson AD (1989) The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *Br J Pharmacol* **96:**291-300.

Griffin BW, Klimko P, Crider JY and Sharif NA (1999) Al-8810; A novel prostaglandin $F_{2\alpha}$ analog with selective antagonist effects at the prostaglandin $F_{2\alpha}$ (FP) receptor. *J Pharmacol Exp Ther* **290:**1278-1284.

Jung J, Hwang SW, Kwak J, Lee SY, Kang CJ, Kim WB, Kim D and Oh U (1999) Capsaicin binds to the intracellular domain of the capsaicin-activated ion channel. *J Neurosci* **19:**529-538.

Kozak KR and Marnett LJ (2002) Oxidative Metabolism Of Endocannabinoids. *Prostaglandins Leukot Essent Fatty Acids* **66:**211-220.

Kozak KR, Crews BC, Morrow JD, Wang LH, Ma YH, Weinander R, Jakobsson PJ and Marnett LJ (2002) Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. *J Biol Chem* **277:**44877-44885.

Kozak KR, Crews BC, Ray JL, Tai HH, Morrow JD and Marnett LJ (2001) Metabolism of prostaglandin glycerol esters and prostaglandin ethanolamides in vitro and in vivo. *J Biol Chem* **276:**36993-36998.

Lawrence RA and Jones RL (1992) Investigation of the prostaglandin E (EP-) receptor subtype mediating relaxation of the rabbit jugular vein. *Br J Pharmacol* **105:**817-824.

Maccarrone M, Attina M, Bari M, Cartoni A, Ledent C and Finazzi-Agro A (2001) Anandamide degradation and N-acylethanolamines level in wild-type and CB1 cannabinoid receptor knockout mice of different ages. *J Neurochem* **78:**339-348.

Maddox YT, Ramwell PW, Shiner CS and Corey EJ (1978) Amide and 1-Amino derivatives of prostaglandins as prostaglandin antagonists. *Nature* **273:**549-552.

Maurelli S, Bisogno T, De Petrocellis L, Di Luccia A, Marino G and Di Marzo V (1995) Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase'. *FEBS Lett* **377:**82-86.

Maxey KM, Johnson JL and Labrecque J (2002) The hydrolysis of bimatoprost in corneal tissue generates a potent prostanoid FP receptor agonist. *Surv Ophthalmol* **47:**S34-S40.

Milne SA, Armstrong RA and Woodward DF (1995) Comparison of the EP receptor subtypes mediating relaxation of the rabbit jugular and pig saphenous veins. *Prostaglandins* **49:**225-237.

Nials AT, Coleman RA, Hartley D and Sheldrick RLG (1991) AH13205 – a novel selective prostanoid EP_2 agonist. *Br J Pharmacol* **102:**24P.

Regan JW, Bailey TJ, Pierce K, Zheng D, Donello J, Kedzie KM, Woodward DF, Fairbairn CE and Gil DW (1994) Molecular cloning and expression of human EP_3 receptors: evidence of 3 variants with differing carboxyl termini. *Br J Pharmacol* **112:**377-385.

Reggio PH (2002) Endocannabinoid structure-activity relationships for interaction at the cannabinoid receptors. *Prostaglandins Leukot Essent Fatty Acids* **66:**143-160.

Resul B, Stjernschantz J, Selen G & Bito L (1997) Structure-activity relationships and receptor profiles of some ocular hypotensive prostanoids. *Surv Ophthalmol.* **41** Suppl 2: S47-52.

Ross RA, Craib SJ, Stevenson LA, Pertwee RG, Henderson A, Toole J and Ellington HC (2002) Pharmacological characterization of the anandamide cyclooxygenase metabolite: prostaglandin E2 ethanolamide. *J Pharmacol Exp Ther* **301:**900-907.

Sharif NA, Williams GW, Kelly CR. (2001) Bimatoprost and its free acid are prostaglandin FP receptor agonists. *Eur J Pharmacol* **432**:211-213.

Ueda N and Yamamoto S (2000) Anandamide amidohydrolase (fatty acid amide hydrolase). *Prostaglandins Other Lipid Mediat* **61:**19-28.

van der Stelt M, Van Kuik JA, Bari M, Van Zadelhoff G, Leeflang BR, Veldink GA, Finazzi-Agro A, Vliegenthart JF and Maccarrone M (2002) Oxygenated metabolites of anandamide and 2-arachidonoylglycerol: conformational analysis and interaction with cannabinoid receptors, membrane transporter, and fatty acid amide hydrolase. *J Med Chem* **45:**3709-3720.

Woodward DF, Krauss AH-P, Chen J, Gil DW, Kedzie KM, Protzman CE, Shi L, Chen R, Krauss HA, Bogardus A, Dinh HTT, Wheeler LA, Andrews SW, Burk RM, Gac T, Roof MB, Garst ME, Kaplan LJ, Sachs G, Pierce KL, Regan JW, Ross RA and Chan MF (2000) Replacement of the carboxylic acid group of prostaglandin $F_{2\alpha}$ with a hydroxyl or methoxy substituent provides biologically unique compounds. *Br J Pharmacol* **130:**1933-1943.

Woodward DF, Krauss AHP, Chen J, Lai RK, Spada CS, Burk RM, Andrews SW, Shi L, Liang Y, Kedzie KM, Chen R, Gil DW, Kharlamb A, Archeampong A, Ling J, Madhu C, Ni J, Rix P, Usansky J, Usansky H, Weber A, Welty D, Yang W, Tang-Liu DD, Garst ME, Brar B, Wheeler LA and Kaplan LJ (2001) The pharmacology of bimatoprost (Lumigan). *Surv Ophthalmol* **45:**S337-S345.

Woodward DF, Krauss AHP, Chen J, Liang Y, Li C, Protzman CE, Bogardus A, Chen R, Kedzie KM, Krauss HA, Gil DW, Kharlamb A, Wheeler LA, Babusis D, Welty D, Tang-Liu DDH, Cherukury M, Andrews SW, Burk RM and Garst ME (2003) Pharmacological Characterization of a Novel Anti-Glaucoma Agent, Bimatoprost (AGN 192024). *J Pharmacol Exp Ther*, **305**:772-785.

Yu M, Ives D and Ramesha CS (1997) Synthesis of prostaglandin E_2 ethanolamide from anandamide by cyclooxygenase-2. *J Biol Chem* **272:**21181-21186.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, Julius D and Hogestatt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400:**452-457.

Footnotes

#These authors contributed equally to this work

*These authors share senior authorship

• Author to whom all correspondence should be addressed

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

Legends to figures

Figure 1. Chemical structures of prostamides D_2, $F_{2\alpha}$ and E_2 and of bimatoprost.

Figure 2. Cat iris sphincter smooth muscle: a comparison of the contractile effects of (a) $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ (b) PGD_2 and prostamide D_2 (c) PGE_2 and prostamide E_2 (d) 11ß-$PGF_{2\alpha}$ and 11ß-prostamide $F_{2\alpha}$. Values are mean ± S.E.M. *n* values are (a) *n* = 6 (b) PGD_2 *n* = 8, prostamide D_2 *n* = 4, (c) PGE_2 *n* = 8, prostamide E_2 *n* = 4 (d) *n* = 4.

Figure 3. Cat recombinant FP receptor (stably transfected): a comparison of effects on intracellular $[Ca^{2+}]$ signaling for (a) $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ (b) PGD_2 and prostamide D_2 (c) PGE_2 and prostamide E_2 (d) 11ß-$PGF_{2\alpha}$ and 11ß-prostamide $F_{2\alpha}$. Values are mean ± S.E.M. *n* = 3 of separate duplicate determinations.

Figure 4. Cat recombinant FP receptor (stably transfected): a comparison of the radioligand binding competition vs. $[^3H]$-17-phenyl $PGF_{2\alpha}$ (5nM) for 17-phenyl $PGF_{2\alpha}$, prostamide D_2, prostamide E_2, and prostamide $F_{2\alpha}$. Values are mean ± S.E.M. *n* = 3 of separate duplicate determinations.

Figure 5. Endothelium-intact rabbit jugular vein: a comparison of the vasorelaxant effects of (a) PGD_2 and prostamide D_2 (b) $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$. Values are mean ± S.E.M. (a) *n* = 5, (b) $PGF_{2\alpha}$ *n* = 7, prostamide $F_{2\alpha}$ *n* = 6.

Figure 6. Effect of prostamide E_2 (PGE_2-EA), $F_{2\alpha}$ ($PGF_{2\alpha}$-EA) and D_2 (PGD_2-EA), with or without pluronic, in HEK-293 transfected with human TRPV1 measured by cytosolic calcium concentration. Changes in $[Ca^{++}]_i$ after treatment with prostamide were monitored with Fluo-3 methylester. Data are expressed as the percent of the effect observed with 4 μM of ionomycin (mean ± S.E.M. of *n* = 3). Error bars are not shown for the sake of clarity and were never higher than 15% of the means.

Figure 7. Effect of prostamides E_2, $F_{2\alpha}$ and D_2 and bimatoprost on FAAH activity. The inhibitory effect of these compounds (50 and 100 μM) were tested on the enzymatic hydrolysis of [^{14}C]anandamide (8 μM , 20000 cpm) by membranes from mouse N18TG2 cells at 37°C for 30 min. Data are expressed as percent of controls and are mean ± S.E.M. of n = 6. Lower concentrations were also tested and were without effect.

Figure 8. Effect of prostamides E_2, $F_{2\alpha}$, D_2 and bimatoprost on anandamide cellular uptake. The lack of any significant inhibitory effect of these compounds (50 μM) on the uptake of [^{14}C]anandamide (4 μM , 10000 cpm) by intact RBL-2H3 basophilic cells for 5 minutes at 37°C is shown. Data are expressed as percent of controls and are mean ± S.E.M. of n = 6. Lower concentrations were also tested and were without effect.

Figure 9. Thin layer chromatography analysis of bimatoprost (0.5 mg) after 4-hour incubation at 37°C with either cat iris homogenate (0.5 mg of protein), or buffer, at pH 7.4. Under the same conditions, 17-phenyl-prostaglandin $F_{2\alpha}$, the possible product of bimatoprost analysis, was found to have an R_f similar to that of the prostaglandin $F_{2\alpha}$ standard shown here.

Figure 10. Cellular uptake of [^{3}H]-prostamide E_2, D_2 and $F_{2\alpha}$ (PGE_2-EA, PGD_2-EA and $PGF_{2\alpha}$ -EA, respectively, 5μM, 30000 cpm) by RBL-2H3 cells (A) and of [^{3}H]-PGE_2-EA and $PGF_{2\alpha}$-EA (5μM, 30000 cpm) by rat brain synaptosomes (B). Cellular and synaptosomal [^{14}C]anandamide uptake (2.5 μM, 10000 cpm) is also shown as a comparison. The radioactivity of [^{3}H]-prostamide found in cells and synaptosomes was measured after incubations carried out at 37 or 4°C. Data are expressed as percent of total radioactivity incubated (mean ± S.E.M. of n = 3). * Denotes statistically significant differences ($P<0.05$ by ANOVA) between 37°C and 4°C incubations. Error bars are not shown for the sake of clarity and were never higher than 20% of the means.

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1. Comparison of the Ca^{2+} signal (FLIPR™) produced by prostamide D_2 and PGD_2, prostamide E_2 and PGE_2, and prostamide $F_{2\alpha}$ and $PGF_{2\alpha}$ using human recombinant prostanoid receptors.

n = 3 of separate duplicate experiments; *n* values for cat iris sphincter are given on the legend to Fig. 2.

Prostanoid Receptor Subtype	Prostamide D_2 EC_{50} [nM]	PGD_2 EC_{50} (nM)	Prostamide E_2 EC_{50} [nM]	PGE_2 EC_{50} [nM]	Prostamide $F_{2\alpha}$ EC_{50} [nM]	$PGF_{2\alpha}$ EC_{50} [nM]
DP	>10,000	12	NA	4332	NA	> 10,000
EP_1	NA	250	848	0.2	>10,000	8.3
EP_2	NA	> 10,000	>10,000	2.5	NA	> 10,000
EP_3	NA	120	123	0.3	NA	7.6
EP_4	NA	2455	>10,000	0.5	NA	6457
FP	NA	13	NA	217	>10,000	5
IP	NA	NA	NA	NA	NA	NA
TP	NA	82	NA	49	NA	126
Cat Iris	499	150	564	260	57	11

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 2. Comparison of the radioligand competition binding afforded by prostamide D_2 and PGD_2, prostamide E_2 and PGE_2, and prostamide $F_{2\alpha}$ and $PGF_{2\alpha}$ at human recombinant prostanoid receptors.

Radioligands were [^{3}H]-PGE_2 ($EP_{1\text{-}4}$), [^{3}H]-17-phenyl $PGF_{2\alpha}$, (FP), and [^{3}H]-U-46619 (TP). *n* = 3 of separate duplicate experiments (NA = no competition at concentrations up to 10^{-5} M).

Prostanoid Receptor Subtype	Prostamide D_2 IC_{50} [nM]	PGD_2 IC_{50} [nM]	Prostamide E_2 IC_{50} [nM]	PGE_2 IC_{50} [nM]	Prostamide $F_{2\alpha}$ IC_{50} [nM]	$PGF_{2\alpha}$ IC_{50} [nM]
hEP_1	>10,000	—	10,000	13	NA	—
hEP_2	NA	14,400	>10,000	27	>10,000	19,500
hEP_3	NA	>10,000	10,000	4.4	>10,000	2273
hEP_4	NA	—	2930	4.8	>10,000	—
hFP	NA	—	> 10,000	—	2015	52
hTP	NA	—	NA	—	>10,000	>10,000

TABLE 3. Comparison of the activities of prostamide $F_{2\alpha}$ with standard receptor selective prostanoids in human platelet, human dermal fibroblast (CRL 1497), and isolated tissue preparations.

n = 4 for all preparations, *n* = 6 for the rabbit isolated jugular vein.

Receptor Subtype	Preparation	Prostamide $F_{2\alpha}$ EC_{50} [nM]	Receptor Selective Prostanoid EC_{50} [nM]
DP	Inhibition of ADP-induced human platelet aggregation	NA	PGD_2 = 79
EP_1	Guinea pig ileum	>10,000	PGE_2 = 6
EP_3	Inhibition of field stimulated guinea pig vas deferens	1585	Sulprostone = 0.2
FP	Relaxation of rabbit jugular vein	2000	$PGF_{2\alpha}$ = 2.8
	CRL 1497 fibroblasts	1586	$PGF_{2\alpha}$ = 25
IP	Inhibition of ADP-induced human platelet aggregation	NA	Carbaprostacyclin = 708
TP	Rat aorta contraction	>10,000	U-46619 = 10

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 4. Hydrolysis of prostamides into the corresponding prostaglandins by brain (prostamide E_2, D_2 and $F_{2\alpha}$), liver (prostamide $F_{2\alpha}$) and lung (prostamide $F_{2\alpha}$) homogenates.

Data (mean ± S.E.M *n* = 6) are subtracted from control hydrolysis (obtained from the same amounts of prostamides incubated with proteins denatured for 10 min at 100°C) and are expressed as percent of total radioactivity incubated (i.e. obtained from the same amounts of prostamides incubated with buffer). The percent hydrolysis of [^{14}C]anandamide under the same conditions is also shown.

	E_2	D_2	$F_{2\alpha}$			AEA		
	Brain	Brain	Brain	Liver	Lung	Brain	Liver	Lung
pH 7.4	1.8 ± 0.8	2.5 ± 0.9	0.4 ± 0.2	2.5 ± 0.3	1.2 ± 0.5	88.9 ± 1.7	90.6 ± 5.3	48.1 ± 1.4
pH 9.0	1.0 ± 0.9	2.7 ± 0.9	0.4 ± 0.2	2.8 ± 0.1	0	93.5 ± 3.2	100.4±1.9	49.1 ± 0.2

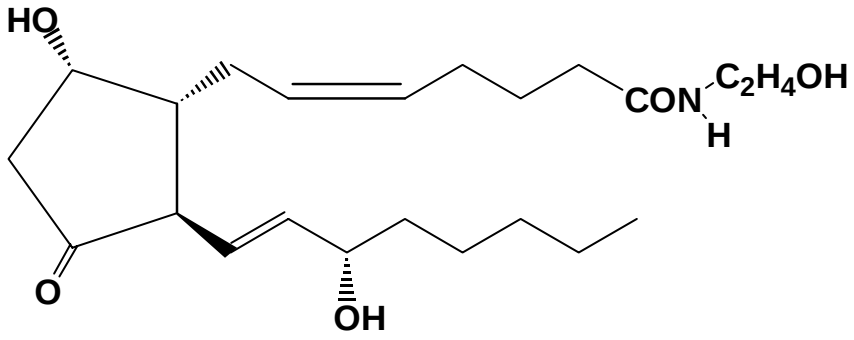
TABLE 5. Hydrolysis of prostamide $F_{2\alpha}$ into the corresponding prostaglandins by cat iris sphincter and ciliary body homogenates.

[^{3}H]-Prostamide $F_{2\alpha}$ (10 μM, 20000 cpm) was incubated with 0.8 mg of ciliary body protein and 0.5 mg of iris protein at pH 7.4 or 9.0 for 4 hours at 37°C. Data (mean ± S.E.M $n = 4$) are subtracted from control hydrolysis (obtained from the same amounts of prostamides incubated with proteins denatured for 10 min at 100°C) and are expressed as percent of total radioactivity incubated (i.e. obtained from the same amounts of prostamides incubated with buffer). N.D. indicates not detected. The percent hydrolysis of [^{14}C]anandamide (AEA, 10 μM, 40,000 cpm, 0.5 mg of protein) under the same conditions and using the same homogenates is shown.

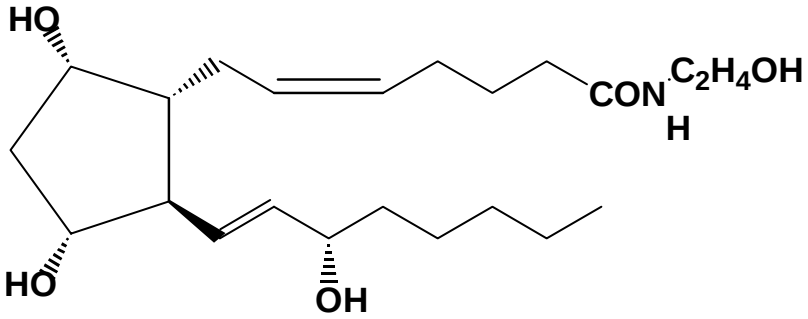
	$F_{2\alpha}$		AEA	
	Ciliary Body	Iris	Ciliary Body	Iris
pH 7.4	N.D.	N.D.	23.5 ± 2.5	6.2 ± 0.1
pH 9.0	N.D.	N.D.	50.0 ± 5.8	46.7 ± 5.6

JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

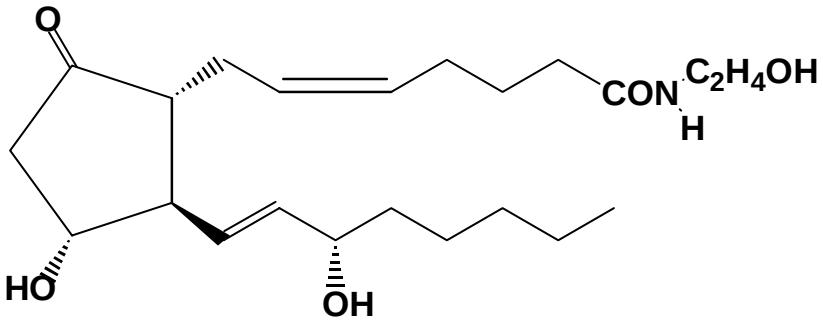
FIGURE 1.



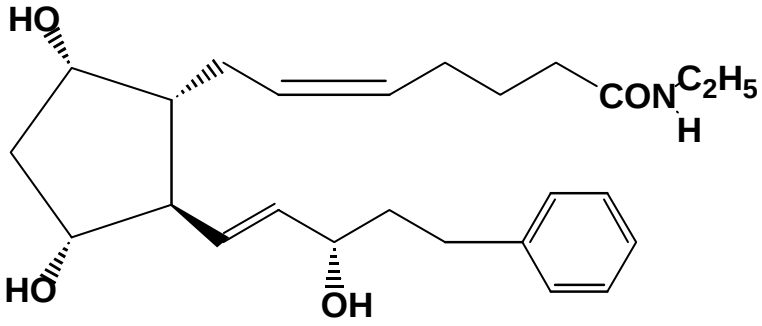
Prostamide D_2



Prostamide $F_{2\alpha}$



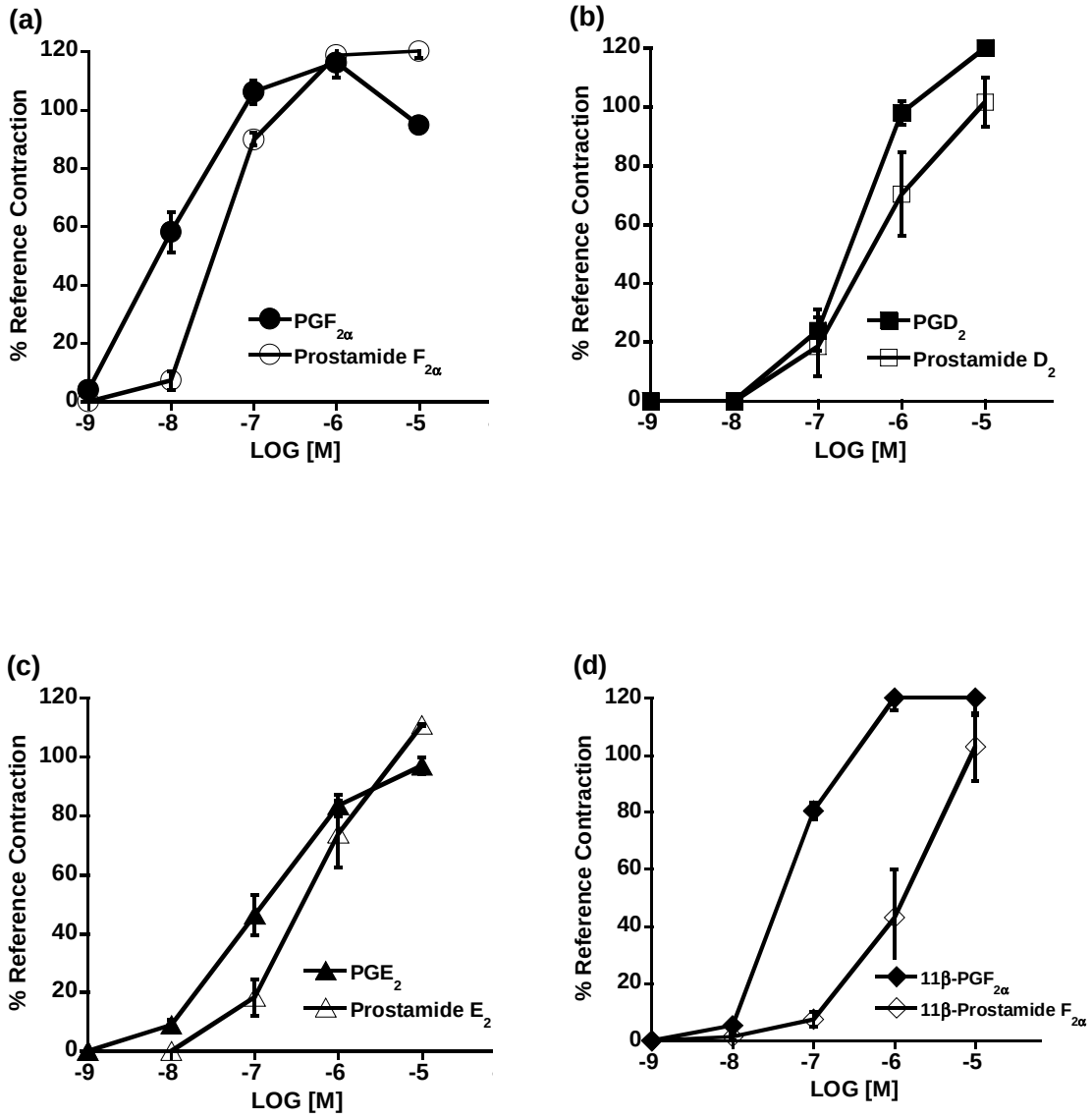
Prostamide E_2



Bimatoprost

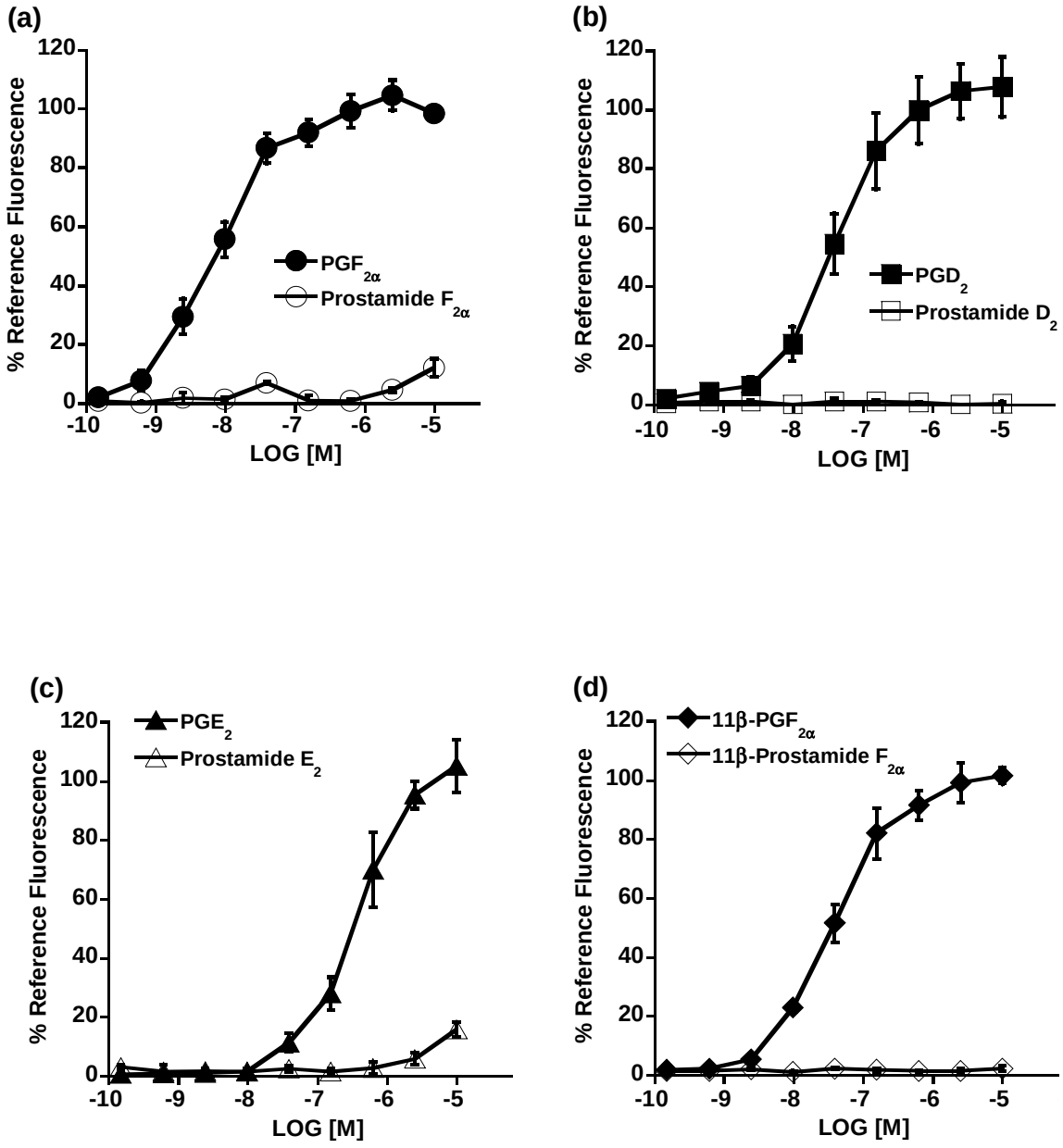
JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 2.



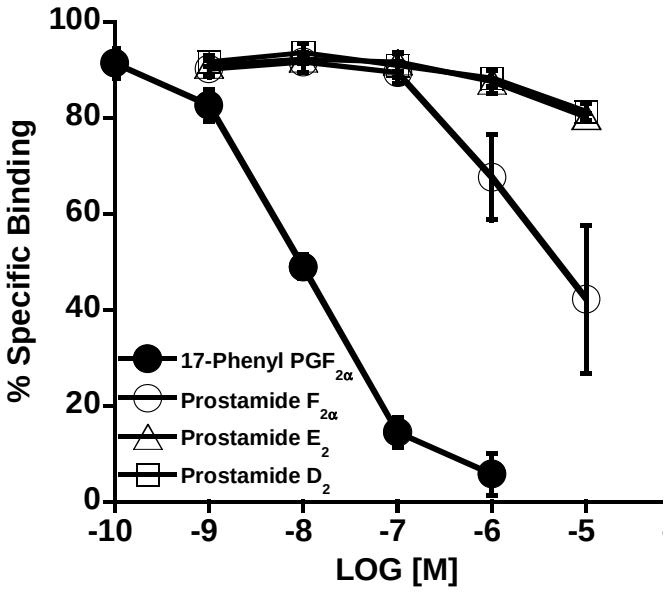
JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 3.



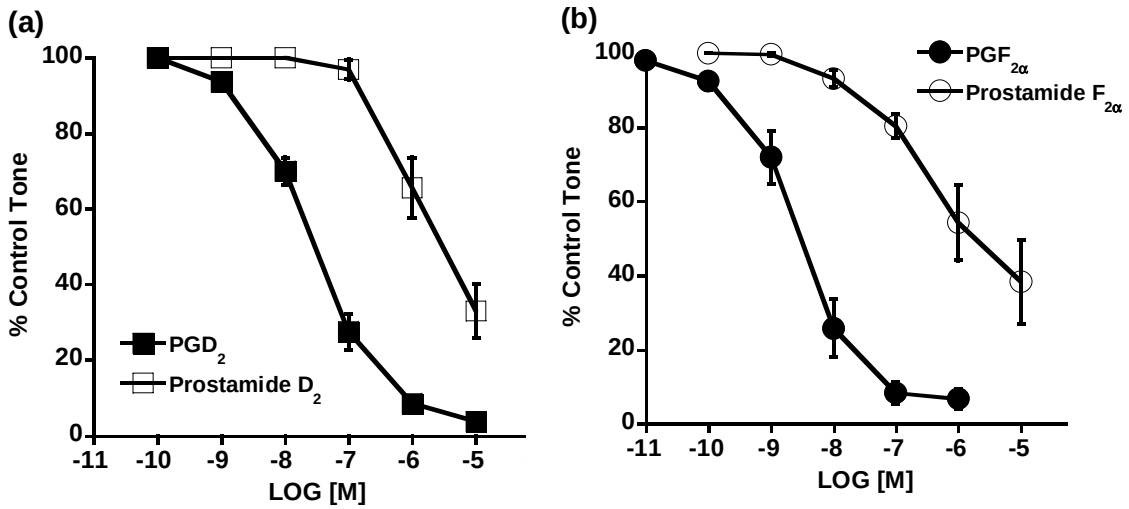
JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 4.



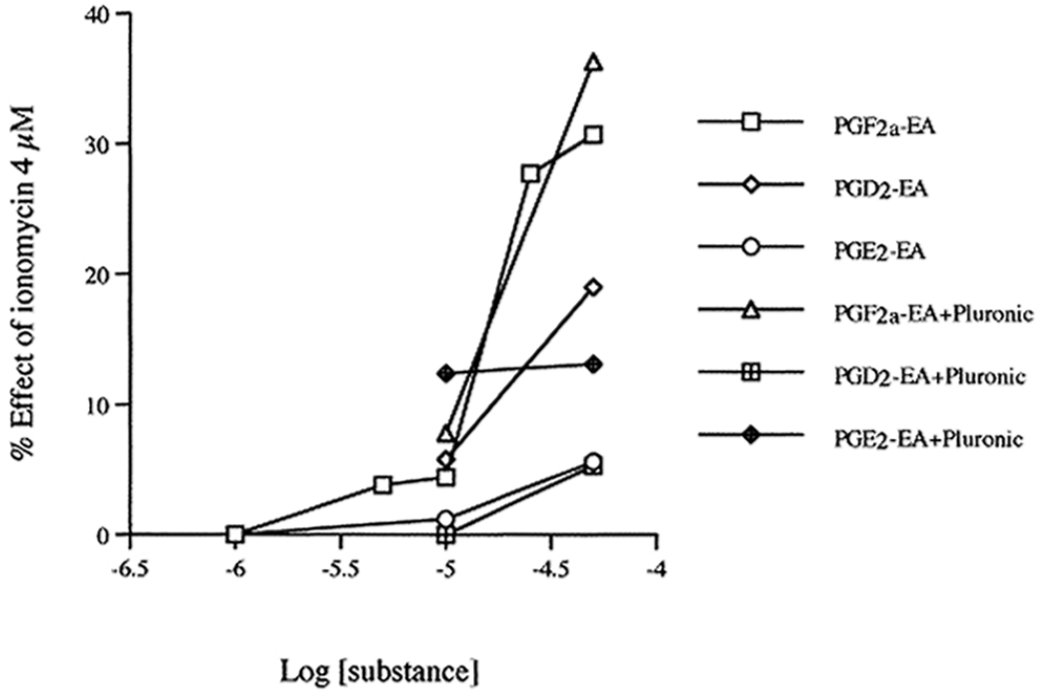
JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 5.



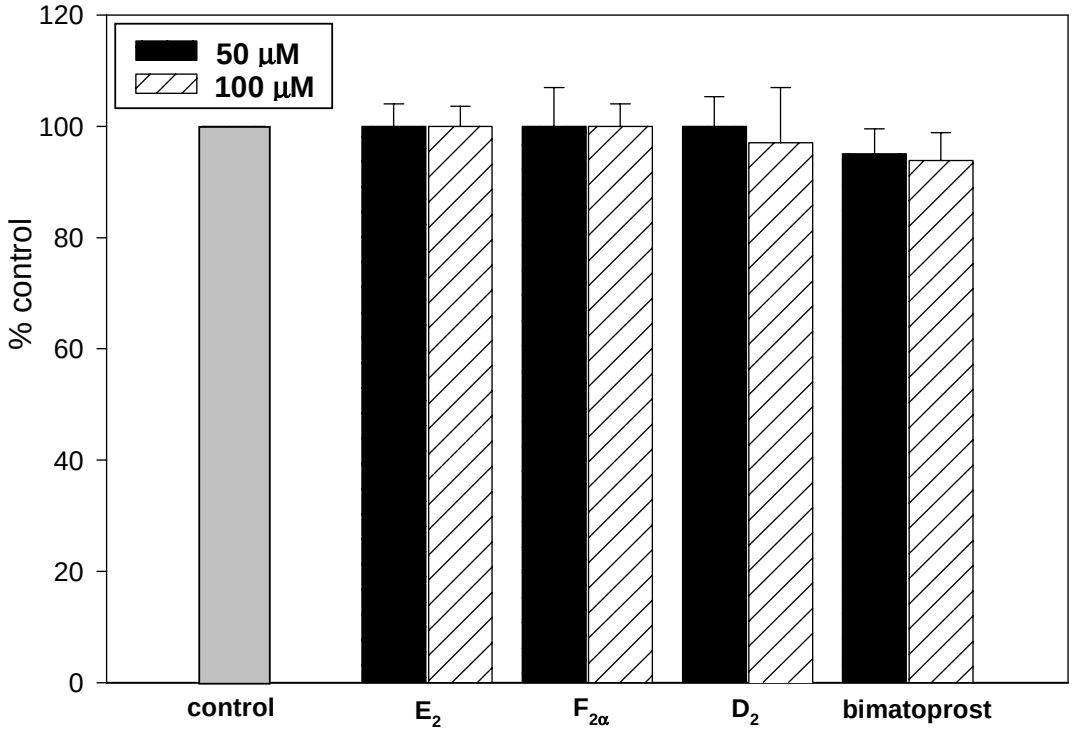
JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 6.



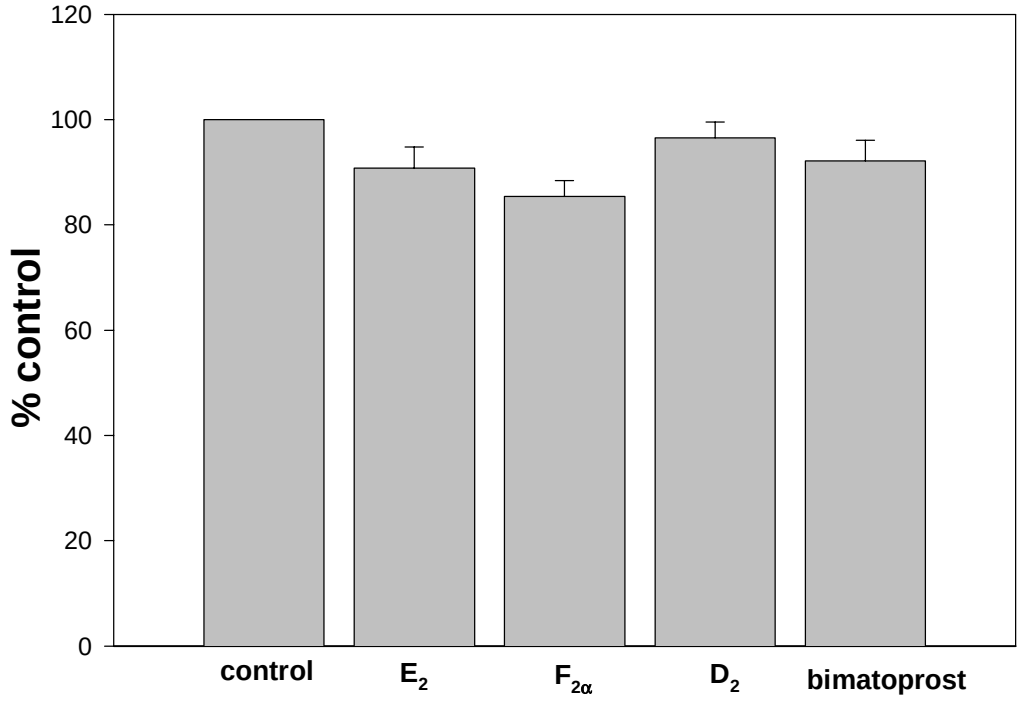
JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 7.



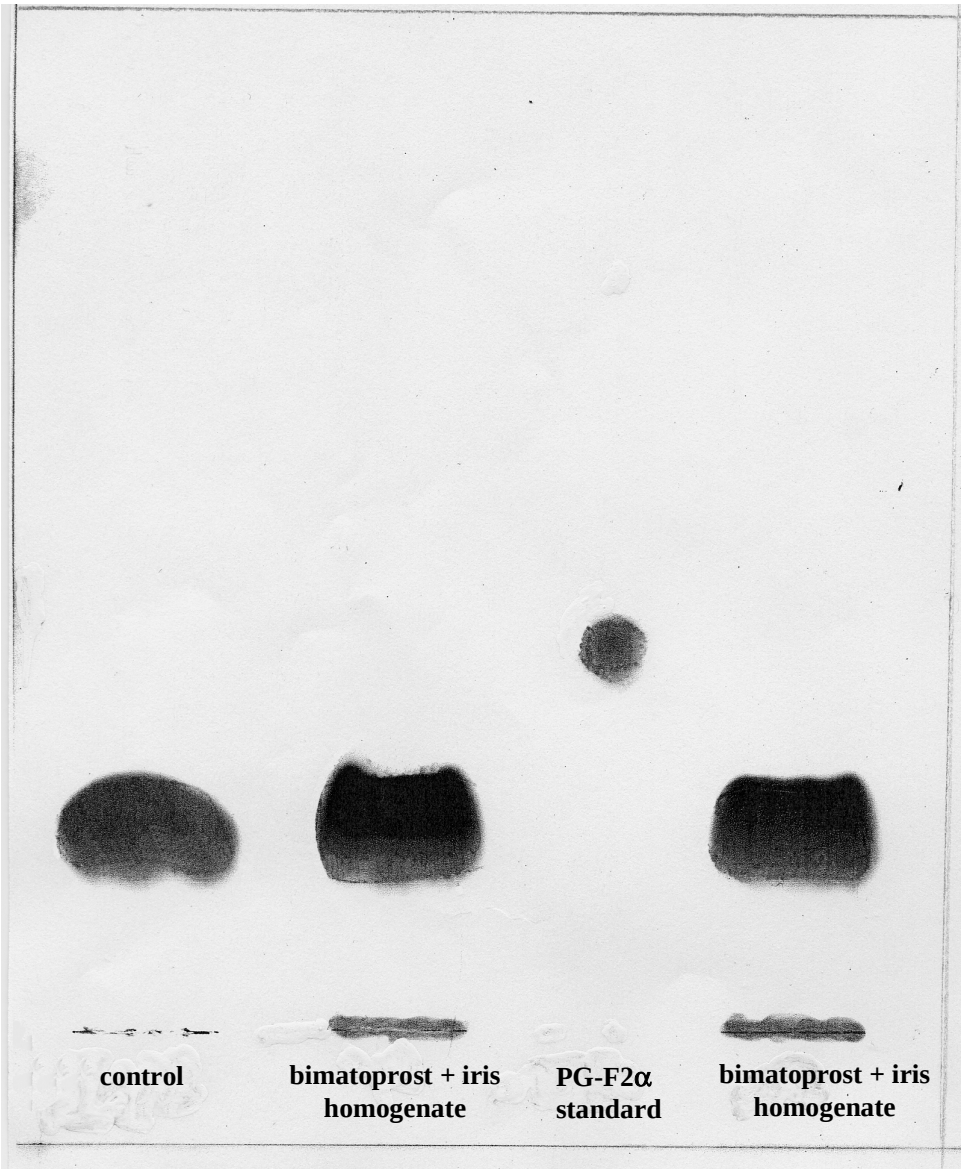
JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 8.



JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

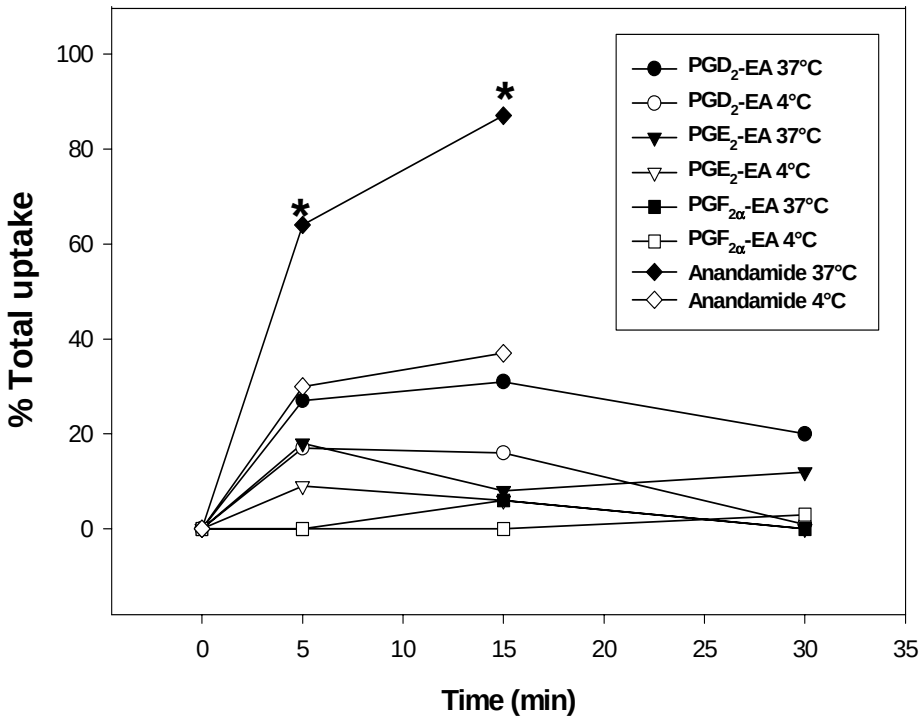
FIGURE 9.



JPET Fast Forward. Published on February 2, 2004 as DOI: 10.1124/jpet.103.061705
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 10.

(a)



(b)