Prostaglandin Ethanolamides (Prostamides): In Vitro Pharmacology and Metabolism


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ABSTRACT

We investigated whether prostaglandin ethanolamides (prostamides) E2, F2α, and D2 exert some of their effects by 1) activating prostanooid receptors either per se or after conversion into the corresponding prostaglandins; 2) interacting with proteins for the inactivation of the endocannabinoid N-arachidonoylthanolamide (AEA), for example fatty acid amide hydrolase (FAAH), thereby enhancing AEA endogenous levels; or 3) activating the vanilloid receptor type-1 (TRPV1). Prostamides potently stimulated cat iris contraction with potency approaching that of the corresponding prostaglandins. However, prostamides D2, E2, and F2α exhibited no meaningful interaction with the cat recombinant FP receptor, nor with human recombinant DP, EP1-4, FP, IP, and TP prostanooid receptors. Prostamide F2α was also very weak or inactive in a panel of bioasays specific for the various prostanooid receptors. None of the prostamides inhibited AEA enzymatic hydrolysis by FAAH in cell homogenates, or AEA cellular uptake in intact cells. Furthermore, less than 3% of the compounds were hydrolyzed to the corresponding prostaglandins when incubated for 4 h with homogenates of rat brain, lung, or liver, and cat iris or ciliary body. Very little temperature-dependent uptake of prostamides was observed after 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 prostanooid receptors, enhancement of AEA levels, or gating of TRPV1 receptors, but possibly to interaction with novel receptors that seem to be functional in the cat iris.

The endocannabinoid N-arachidonoylthanolamine (anandamide, AEA) is inactivated via a two-step mechanism, including reuptake 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-arachidonamides, 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 (COX)-2 (Kozak and Marnett, 2002). In particular, COX-2, but not COX-1, recognizes AEA and catalyzes its conversion to prostaglandin (PG) endoperoxide ethanolamides, opening the way to the formation of prostaglandin E2, D2, and F2α 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 CB1 and CB2 receptors (Berglund et al., 1999) and 2 to 3 orders of magnitude less active than the corresponding PGs

ABBREVIATIONS: AEA, anandamide; FAAH, fatty acid amide hydrolase; COX, cyclooxygenase; CB, cannabinoid; PG, prostaglandin or prostanooid; TRPV1, vanilloid receptor type 1; HEK, human embryonic kidney; TLC, thin layer chromatography; EBNA, Epstein Barr nuclear antigen; FLIPR, fluorometric imaging plate reader; HA, hemagglutinin; HBSS, Hanks’ balanced salt solution; U-46619, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F2α; SQ29548, [1S(1α,2α(2Z),3α,4α)]-7-[3-[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2,2.1]hept-2-yl]-5-heptanoic acid.
in both binding and functional assays for the several prosta-
noid receptors known to date (Woodward et al., 2001, 2003; 
Ross et al., 2002). For example, prostamide E2 is 100- to 
1000-fold less potent than PGE2 in binding assays involving 
human EP1, EP2, EP3, and EP4 receptor-containing mem-

branes; about 100-fold less potent than the free acid in func-
tional assays of EP3 and EP4 receptors; but surprisingly only 
15 times less potent than PGE2 in EP2 receptor-mediated 
relaxation of the guinea pig trachea (Ross et al., 2002). The 
17-phenyl-derivative of prostamide F2α, known as bimato-
prost (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 prosta-
mides F2α and D2 or of thromboxane and prostacyclin eth-
anolamides. The possibility that prostamides interact with 
another molecular target such as AEA, the vanilloid receptor 
type-1 (TRPV1) (Zygmun et al., 1999; Di Marzo et al., 2002), 
has been investigated only for prostamide E2 (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 
PG inactivation (Kozak et al., 2001), but the possibility that 
these compounds were substrates for FAAH or other ami-
dases was not assessed. This is not a trivial issue because 
tissue-specific hydrolysis of prostamides to the corresponding 
prostaglandins might explain, for example, why prostamides 
F2α and E2 exhibit potency comparable with the correspond-
ing prostaglandins in only a few assays of FP and EP2 recep-
tor activity (Woodward et al., 2001; Ross et al., 2002). Fur-
thermore, if prostamides were good substrates for FAAH, 
they might be capable of increasing the amounts of endoge-
nous 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, 
because this also might lead to an enhancement of extracel-

ular AEA levels.

In the present study, we investigated whether prostamides 
are capable of directly activating the TRPV1 receptor, or 
whether they serve as substrates for FAAH or the AEA 
membrane transporter. Furthermore, we investigated 
whether these compounds can be 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 synaptic 
vesicles or intact RBL-2H3 cells. Finally, we assessed the 
activity of all prostamides, in comparison with the corre-
sponding prostaglandins, in two preparations expressing 
prostanoid FP receptors, as well as at cat recombinant FP 
receptors and human DP, EP1-4, 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 molec-
ular targets.

Materials and Methods

Materials

The 1-ethanolamides of PGD2, PGE2, PGF2α, and 11β-PGF2α were 
synthesized by Allergan, Inc. (Irvine, CA) or purchased from Cayman 
Chemical (Ann Arbor, MI). PGD2, PGE2, and PGF2α, 11β-PGF2α, 
fluprostenol, carbaprostacyclin, U-46619, 17-phenyl PGF2α, and sul-
prostone were purchased from Cayman Chemical. PGF2α-1-CON 
(CH3)2 was synthesized at Allergan, Inc. [3H]PGE2 (specific activity 
165 Ci mmol⁻¹); [3H]prostamides D2, E2, and F2α (specific activity 
80 Ci mmol⁻¹); and [3H]17-phenyl PGF2α (specific activity 85 Ci 
mmol⁻¹) were obtained from Amersham Biosciences UK Ltd. 
(Cardiff, UK). [3H]SQ29548 (specific activity 41.5 Ci mmol⁻¹) was 
purchased from PerkinElmer Life Sciences (Boston, MA). [14C]AEA 
(specific activity 5 Ci mmol⁻¹) was synthesized in our laboratory as 
described previously (Bisogno et al., 1997).

Intracellular Calcium Concentration Assay

Human embryonic kidney (HEK)-293 cells overexpressing the hu-
man TRPV1 receptor were a kind gift from John Davis (GlaxoSmith-
Kline, Harlow, UK). Cells were grown as monolayers in minimum 
esential medium supplemented with nonessential amino acids, 10% 
fetal calf serum, and 0.2 mM glutamine and maintained under 95%, 
5% O2/CO2 at 37°C. The effect of substances on [Ca2⁺]i was deter-
mined by using Fluo-3, a selective intracellular fluorescent probe for 
Ca2⁺. One day before experiments cells were transferred into six-
well dishes coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO)
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and grown in the culture medium mentioned above. On the day of the experiment, the cells (50–60,000/well) were loaded for 2 h at 25°C with 4 μM Fluo-3 methylester (Molecular Probes, Eugene, OR) in dimethyl sulfoxide containing 0.04% Pluronic. 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 (LS50B; PerkinElmer Life Sciences) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25°C (λEX = 488 nm, λEM = 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 (EC50). The efficacy of test compounds at various concentrations. Data are expressed as the ratio of [14C]AEA (10 μM; 20,000 cpm) to the radioactivity of the aqueous phase, which contains [14C]ethanolamine produced from [14C]AE hydrolysis, was measured directly by a beta-counter.

Bimatoprost (0.5 μg) was incubated with 0.5 to 1 mg of rat brain, cat ciliary body, and iris protein (or without, for the control reaction) for 4 h at 37°C in 50 mM Tris buffer, pH 7.4. The reaction mixture was treated and extracted under the same conditions previously described, with bimatoprost and PGF2α 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 [3H]prostamide E2, D2, or F2 (5 μM; 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 et al. (2001) and were incubated for different intervals of time (0, 5, 10, and 20 min) at 37 or 4°C with prostamide E2 or F2 (5 μM; 30,000 cpm). Cellular and synaptosomal uptake of [3H]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 plated on ice and both the incubation media and cells or synaptosomes were separately extracted with chloroform/methanol/[14C]ethanolamine produced from [14C]AE hydrolysis, was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of CHCl3/CH3OH 2:1 (by volume).

**Inhibition Studies of Prostanoids 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 [3H]AEA (8 μM; 20,000 cpm) in 50 mM Tris-HCl, pH 9, for 30 min at 37°C. [3H]Ethanolamine produced from [3H]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of CHCl3/CH3OH 2:1 (by volume).

**Inhibition Studies of Prostamides on AEA Cellular Uptake**

The effect of compounds on the uptake of [3H]AEA by intact rat basophilic leukemia (RBL-2H3) cells was studied by using 4 μM (10,000 cpm) of [3H]AEA as described previously (Bisogno et al., 1997). Cells were incubated with [3H]AEA for 5 min at 37°C, in the presence or absence of the inhibitors (50 μM). Residual [3H]AEA in the incubation media after extraction with CHCl3/CH3OH 2:1 (by volume), 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 [3H]AEA disappeared from the medium of RBL-2H3 cells is found mostly (>90%) as unmetabolized [3H]AEA in the cell extract. Non specific binding of [3H]AEA to cells and plastic dishes was determined in the presence of 100 μM AEA and was never higher than 30%.

**Metabolic Studies with [3H]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 800g, and the protein concentration of each supernatant was determined by the Bradford method. Ten micromolar (20,000 cpm) [3H]prostamide D2, E2, or F2, was incubated for 4 h at 37°C in 50 mM Tris buffer, pH 7.4 or 9.0, with 0.5 to 1 mg of protein. The reaction mixture was then acidified to pH 3.0 with HCl (1 N) and extracted three times with ethyl acetate (2 volumes). Control incubations were carried out in the same conditions but without homogenates. A control for each experiment was also carried out using denatured proteins (inactivated at 100°C for 10 min). The solvent was then evaporated under nitrogen and then brought up in 80 μl of 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/NH4OH for prostamide D2 or F2, or with 75:25:1 chloroform/methanol/NH4OH for prostamide E2. TLC plates were then visualized by brief exposure to iodine vapors and scanned for radioactive bands. [14C]AEA (10 μM; 40,000 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 volume of chloroform/methanol (2:1) (by volume) was added, the mixture vortexed, and the radioactivity of the aqueous phase, which contains [14C]ethanolamine produced from [14C]AEA hydrolysis, was measured directly by a beta-counter.

**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 7E). The organ baths contained Krebs’ solution maintained at 37°C and gassed with 95% O2, 5% CO2 to give a pH of 7.4. The Krebs’ solution had the following composition: 118.0 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.9 mM CaCl2, 1.18 mM MgSO4, 25.0 mM NaHCO3, 11.7 mM d-glucose, and 0.001 mM NaH2PO4.

**Cat Iris**

Adult domestic cats were euthanized by intravenous overdose of sodium pentobarbitual (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 washout and return to baseline tension. The response to 10−5 M PGF2α was determined at the beginning and end of each experiment and between dose-response curves as a reference.

**Endothelium-Intact Rabbit Jugal Vein**

New Zealand Albinor rabbits of either sex, weighing 2 to 4 kg, were injected with 1000 U of heparin into the marginal ear vein and then euthanized by CO2 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- to 4-mm length was suspended between two metal hooks 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 washout and return to baseline tension. The response to 10−5 M PGF2α was determined at the beginning and end of each experiment and between dose-response curves as a reference.
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^{-2} M PGE_{2} at the end of each dose-response curve as a reference standard. A recovery period of 30–50 min was allowed after washout of the tissues. Relaxant activity was calculated as percentage 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-h equilibration period, a standard dose-response to PGE_{2} was obtained in a noncumulative manner with 30-min washout periods between individual doses. Subsequently, graded doses of test compounds were added noncumulatively. A maximal dose of PGE_{2} (10^{-8} 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 percentage of the 10^{-8} 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 20-V pulses of 1-ms duration, each individual pulse separated by 100 ms. Electrical current was generated by a Grass S48 stimulator and distributed to the individual organ baths with a Stimu-splitter II (Med-Lab Instruments, Loveland, CO), using glass tissue supports with stimulating platinum electrodes in parallel orientation to the tissues (Radnoti Glass Technology, Monrovia, CA). After stabilization of the twitch response, PGE_{2} was applied cumulatively. Investigational compounds in parallel orientation to the tissues (Radnoti the individual organ baths with a Stimu-splitter II (Med-Lab Instruments, Loveland, CO), using glass tissue supports with stimulating platinum electrodes in parallel orientation to the tissues (Radnoti Glass Technology, Monrovia, CA). After stabilization of the twitch response, PGE_{2} was applied cumulatively. Investigational compounds were then evaluated in a cumulative manner. PGE_{2} was reapplied 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 percentage of 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- to 8-mm length were prepared. Each segment was mounted under 2.0-g tension. The tissue preparations were allowed to equilibrate for 1 h 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. Thirty to 45 min was allowed for tissue recovery after washout of each drug.

**Cell Studies**

**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. The blood was centrifuged at 1000 rpm for 15 to 20 min to obtain platelet-rich plasma. Then, 4.5 μl of prostaglandin solution or vehicle was added to 450 μl of platelet-rich plasma and incubated for 2 min at 37°C in a Payton aggregometer and observed for any aggregatory activity. ADP (2 x 10^{-8} M) (final concentration) was then added to induce full aggregation. Inhibition of aggregation was calculated as the percentage of difference between aggregation evoked by 2 x 10^{-8} M ADP in the absence and presence of drug. Aggregatory activity was calculated as the percentage of aggregation in response to the prostamide or prostanoïd relative to the aggregation induced by 2 x 10^{-6} M ADP. Standard aggregatory responses to 2 x 10^{-8} M ADP alone were performed at the beginning and end of each experiment.

**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 pCPE4 (Invitrogen, Carlsbad, CA). The pCPE4 vector contains an Epstein Barr virus origin of replication, which permits episomal replication in primate cell lines expressing Epstein Barr virus nuclear antigen (EBNA-1). It also contains a hygromycin resistance gene that is used for eukaryotic selection. The cells used for stable transfection were HEK-293 that were transfected with and express the EBNA-1 protein. These HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 250 μg ml^{-1} G418 (Invitrogen), and 200 μg ml^{-1} gentamicin or penicillin/streptomycin. Selection of stable transfectants was achieved with 200 μg ml^{-1} hygromycin, the optimal concentration being determined by previous hygromycin kill curve studies. For prostanoid receptor transfection, the cells were seeded into six-well plates at a density of 10^{5}well the day before transfection. FuGENE 6 transfection agent (Roche Diagnostics, Indianapolis, IN) was diluted in OPTI-MEM (Invitrogen). The pCPE4 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 of pCPE_{4} vector-prostanoid receptor cDNA. This was followed by 2-d 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 six-well plate and then expanded. Cells were maintained under continuous hygromycin selection as described above until use.

**Human Recombinant EP_{3} and TP Receptors: Binding Studies.** Plasmids encoding the human EP_{3}D isoform (Bogan 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 using the lipofectin method, according to the manufacturer’s instructions (Invitrogen). For radioligand binding studies, cells were harvested 2 d after transfection.

**Radioligand Binding.** Radioligand binding studies using plasma membrane fractions prepared from cells stably expressing the cat or human receptor were performed as follows. 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 needed to achieve a 40-ml volume in the centrifuge tubes. TME is composed of 50 mM Tris base, 10 mM MgCl_{2}, and 1 mM EDTA; pH 7.4 was achieved by adding 1 N HCl. The cell homogenate was centrifuged at 19,000 rpm for 30 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- or 200-μl volume. Binding was determined in duplicate for 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 Inc., Gaithersburg, MD). Competition studies were performed using a final concentration of 2.5 or 5 nM [3H]PGE_{2} (EP_{1}, EP_{2}, and EP_{4} receptors) or 5 nM [3H]17-phenyl PGF_{2a} (FP receptors). Nonspecific binding was determined with 10^{-5} M 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 s 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- or 200-μl volume. Competition binding versus [3H]PGE_{2} at EP_{3D}, receptors or [3H]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 Inc.). Competition studies were performed using a final concentration of 5 nM [3H]PGE2, or 10 nM [3H]PGF2α, and nonspecific binding was determined with 10−5 M respective unlabeled prostanoitd.

Human DP Receptor Luciferase Reporter Assay for hDP-HEK 293/EBNA. Stable DP receptor transfectants were prepared as previously described above for EP1, EP2, EP3, FP and TP receptors. For the luciferase reporter assay, 5 × 104 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 h 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. Twenty microliters of soluble extracts was 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 with control.

Human IP Receptor Luciferase Reporter Assay for hIP-HEK 293/EBNA. Stable IP receptor transfectants were prepared as described previously. Cells for the luciferase reporter assay were prepared as follows. hIP-HEK 293/EBNA cells were seeded in a 24-well plate 24 h before transfection. The CRE-luciferase reporter plasmid (Stratagene) was transiently transfected into the above-mentioned 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 h 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 with control.

Human Recombinant DP, EP1, EP2, EP3, and EP4 Receptors: Ca2+ Signaling (Fluorometric Imaging Plate Reader, FLIPR) Studies. The use of chimeric G protein cDNAs allowed responses to Gs- and Gi-coupled receptors to be measured as a Ca2+ signal. Prostanoid DP, EP1, and EP2 receptor cDNAs were cotransfected with chimeric Gαs cDNA containing a hemagglutinin (HA) epitope. The pseudoadranoid EP1 receptor was cotransfected with chimeric Gq/11-HA. Gqsα and Gqα5 chimera cDNAs (Molecular Devices Corp., Sunnyvale, CA) were also cloned into pCPEB 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 described previously. Because the Gqs and Gq chimerics contain an HA epitope, protein expression may be detected by specific antibody. In Western blot experiments, anti-HA-peroxidase and a mouse monoclonal antibody (clone 12 CA5) were used.

Human Recombinant EP1, IP, DP, and TP Receptors: FLIPR Studies. Stable transfectants were obtained as described for radioligand binding studies. Briefly, pCPEB 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.

Ca2+ Signal Studies Using FLIPR. Cells were seeded at a density of 5 × 104 cells/well in Biocoat poly-l-lysine-coated blackwall, clear-bottom 96-well plates (BD Biosciences, Franklin Lakes, NJ) 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 to 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 100 μl of 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- to 570-nm bandwidth emission filter (FLIPR: Molecular Devices Corp.). 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 three 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 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 PGD2, PGE2, PGF2α, and 11β-PGF2α, were compared with the activities of the corresponding ethanamide analogs (whose chemical structures are shown in Fig. 1) in the cat isolated iris sphincter preparation. These data are depicted in Fig. 2. Prostamide D2 was the most potent prostamide, with an EC50 value of 57 nM. Prostamides D2 and E2 were essentially equipotent but were approximately 10-fold less active than prostamide F2α, with EC50 values of 499 and 564 nM, respectively (Fig. 2). The corresponding free acids of prostamides D2, E2, and F2α were only marginally more active than the prostamides: EC50 (nM) values were PGF2α = 11, PGD2 = 150, and PGE2 = 260. A marked separation between the activity of 11β-PGF2α and the corresponding ethanamide was apparent: EC50 values were 11β-PGF2α = 54 and 11β-ethanamide = 1827. The activity of PGF2α-1-CON (CH3) was also evaluated in the cat iris and an EC50 value of 450 nM was obtained (data not shown).

Effect of Prostamides on the Cat Recombinant FP Receptor. Because 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 seem to be the case. Prostamide interaction at the feline FP receptor was no more than residual compared with PGF2α activity (Fig. 3). This was confirmed for prostamides D2, E2, and F2α, by radioligand binding (Fig. 4). The activity of the natural PGs at cat recombinant FP receptors showed the typical potency rank order PGF2α > PGD2 > PGE2 that has been described previously for other preparations (Coleman et al., 1984). EC50 values (nanomolar) at the cat recombinant FP receptor were PGF2α = 6.8, 11β-PGF2α = 37; PGD2 = 40, and PGE2 = 396; all prostamides >10,000. PGF2α-1-CON (CH3) exhibited no meaningful activity at the cat recombinant FP receptor (EC50 > 10,000; data not shown).

Effect of Prostamides on the Precontracted Rabbit Jugular Vein. The relaxant effects of prostamides D2 and F2α, on the precontracted rabbit jugular vein are compared with those of PGD2 and PGF2α, in Fig. 5. In marked contrast
to the cat iris sphincter (Fig. 2a), a substantial difference between the potency of PGF_2α and prostamide F_2α, was apparent in the precontracted rabbit jugular vein preparation. Similarly, PGD_2 was approximately 2 orders of magnitude more potent than prostamide D_2 (Fig. 5a). EC50 values (nanomolar) were obtained as follows: PGF_2α/H9251 2.8, prostamide F_2α/H9251 2000, PGD_2/H11005 28, and prostamide D_2/H11005 3060. The difference in potency between prostamide D_2 and PGD_2 is similar to the difference between PGE_2 and prostamide E_2 reported previously by Ross et al. (2002).

**Effect of Prostamides on Recombinant and Native Prostanoid Receptors.** The pharmacology of prostamides was further compared with the natural PGs in functional and radioligand binding competition studies using 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 with relative activities in the cat iris preparation. Prostamides D_2 and F_2α were essentially inactive in terms of stimulating human recombinant receptors. Prostamide E_2 was more active than prostamides D_2 and F_2α. A measurable EC50 value was obtained for prostamide E_2 at the EP_1 receptor (848 nM) and the EP_3 receptor (123 nM); PGE_2, however, had potent activities at EP receptors (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_1-4, FP, or TP receptors. Prostamide E_2 exhibited weak affinity for the EP_4 receptors. Prostamide F_2α exhibited weak affinity for the FP receptor and the EP_2-4 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_2α. PGD_2 and carba-prostacyclin served as positive controls and behaved as potent agonists at DP and

---

**Fig. 2.** Cat iris sphincter smooth muscle: a comparison of the contractile effects of PGF_2α and prostamide F_2α (a), PGD_2 and prostamide D_2 (b), PGE_2 and prostamide E_2 (c), and 11β-PGF_2α and 11β-prostamide F_2α (d). Values are mean ± S.E.M. n values are n = 6 (a), PGD_2 n = 8, prostamide D_2 n = 4 (b), PGE_2 n = 8, prostamide E_2 n = 4 (c), and n = 4 (d).
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 overexpressing the human TRPV1 receptor. Previous studies have shown that this assay is more sensitive than other TRPV1 assays because, for example, the EC$_{50}$ values for AEA and capsaicin in this assay are usually at least 1 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$_{50}$ of 15.0$\pm$2.4 M and a maximal effect that was 31$\pm$2% of the maximal effect observable with 4 M ionomycin (mean$\pm$S.E.M., $n=3$) (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 M concentration (data not shown). Under the same conditions, AEA and capsaicin exhibit EC$_{50}$...
values of $540 \pm 120$ and $35 \pm 8$ nM, respectively. The effect of prostamide $F_{2\alpha}$ (50 $\mu$M) was blocked by the TRPV1 antagonist capsazepine (1 $\mu$M, from 31 $\pm$ 2 to 5 $\pm$ 2% of the maximal effect of ionomycin; mean $\pm$ S.E.M., $n = 3, P < 0.05$); no effect was observed in nontransfected HEK-293 cells (data not shown). Because 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 because prostamide $F_{2\alpha}$ seemed 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, although 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 the 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 $\mu$M (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 $\mu$M (Fig. 8).

Metabolic Stability of Prostamides in Homogenates from Various Tissues. The enzymatic hydrolysis of the prostamides was assessed by using the corresponding $^3$H-labeled 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-labeled corresponding prostaglandins were formed (Tables 4 and 5). Under the same conditions, 50–100% of $[^{14}C]$AEA was hydrolyzed to $[^{14}C]$ethanolamine (Tables 4 and 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-labeled 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. 10, a and b). However, unlike RBL-2H3 cells, the very modest

<table>
<thead>
<tr>
<th>Prostanoid Receptor Subtype</th>
<th>Prostamide $D_2$ $EC_{50}$ (nM)</th>
<th>Prostaglandin $D_2$ $EC_{50}$ (nM)</th>
<th>Prostamide $E_2$ $EC_{50}$ (nM)</th>
<th>Prostaglandin $E_2$ $EC_{50}$ (nM)</th>
<th>Prostamide $F_2\alpha$ $EC_{50}$ (nM)</th>
<th>Prostaglandin $F_2\alpha$ $EC_{50}$ (nM)</th>
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<tr>
<td>DP</td>
<td>$&gt;10,000$</td>
<td>12</td>
<td>NA</td>
<td>4332</td>
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</tr>
<tr>
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<td>NA</td>
<td>250</td>
<td>848</td>
<td>0.2</td>
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<td>EF$2$</td>
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<td>123</td>
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<td>EF$4$</td>
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<td>6,457</td>
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<td>FP$1$</td>
<td>NA</td>
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<td>NA</td>
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<tr>
<td>TP</td>
<td>NA</td>
<td>82</td>
<td>NA</td>
<td>49</td>
<td>NA</td>
<td>126</td>
</tr>
<tr>
<td>Cat Iris</td>
<td>499</td>
<td>150</td>
<td>564</td>
<td>260</td>
<td>57</td>
<td>11</td>
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</table>

NA, not active.
uptake by synaptosomes was statistically significant ($P < 0.05$). Under the same conditions, $[^{14}C]AEAs$ was taken up by the cells much more efficaciously (Fig. 10, a and b).

**Discussion**

We present evidence that 1) prostamides $E_2$, $D_2$, and $F_{2a}$ have very little, if any, affinity for prostanoid receptors; 2) prostamide $F_{2a}$ potently contracts the feline iris sphincter independent from FP receptors; 3) prostamide $F_{2a}$, and much less so prostamides $E_2$ and $D_2$, directly activate the TRPV1 receptor, albeit at concentrations higher than 10 mM and with low efficacy compared with capsaicin, or AEA; 4) prostamides $E_2$, $D_2$, and $F_{2a}$ 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; and 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 CB$_1$ and CB$_2$ receptors (Berglund et al., 1999), support the
hypothesis that prostamides $E_2$ and $F_2$ might 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 $F_2$ (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 $F_2$, namely, the cat iris sphincter. The results were unexpected. The potencies of prostamides $D_2$, $E_2$, and $F_2$ 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

TABLE 4
Hydrolysis of prostamides into the corresponding prostaglandins by brain (prostamide $E_2$, $D_2$, and $F_2$), liver (prostamide $F_2$), and lung (prostamide $F_2$) homogenates

<table>
<thead>
<tr>
<th></th>
<th>$E_2$</th>
<th>$D_2$</th>
<th>$F_2$</th>
<th>$AEA$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>pH 7.4</td>
<td>1.8 ± 0.8</td>
<td>2.5 ± 0.9</td>
<td>0.4 ± 0.2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>1.0 ± 0.9</td>
<td>2.7 ± 0.9</td>
<td>0.4 ± 0.2</td>
<td>2.8 ± 0.1</td>
</tr>
</tbody>
</table>

Fig. 7. Effect of prostamides $E_2$, $F_2$, 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; 20,000 cpm) by membranes from mouse N18TG2 cells at 37°C for 30 min. Data are expressed as percentage of controls and are mean ± S.E.M. of $n = 6$. Lower concentrations were also tested and were without effect.

Fig. 8. Effect of prostamides $E_2$, $F_2$, and $D_2$ 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; 10,000 cpm) by intact RBL-2H3 basophilic cells for 5 min at 37°C is shown. Data are expressed as percentage of controls and are mean ± S.E.M. of $n = 6$. Lower concentrations were also tested and were without effect.
sphincter seems distinctly sensitive to prostamides D₂, E₂, and F₂α, 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 PGF₂α amides were 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 PGF₂α, analogs with PGF₂α, replacement of the -COOH group of PGF₂α with a -CONH₂ moiety produced about a 300-fold reduction in potency. The monomethyl and dimethyl amide analogs of PGF₂α 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 PGF₂α analogs containing an amido moiety are essentially equipotent with PGF₂α in contracting the cat iris sphincter. Antagonist properties have been claimed for PGF₂α 1-CON (CH₃)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 PGF₂α 1-dimethylamide are arguably deficient because the PGF₂α dose range used was too narrow (Maddox et al., 1978). A recent study claimed that PGF₂α 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 PGD₂- and PGE₂-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, EP₂, and EP₄ 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 very different from those obtained in the cat iris sphincter. In the jugular vein, prostamides were more than 2 orders of magnitude less potent than the corresponding prostaglandins. These results are essentially consistent with a previous report, where PGE₂ was found to be about 200 times more potent than prostamide E₂ in the rabbit jugular vein (Ross et al., 2002).

Prostaglandins D₂, E₂, and F₂α were also compared with 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 whether 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 PGD₂, PGE₂, and PGF₂α 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 (Zygmun et al., 1999). Furthermore, stimulation of TRPV1 receptors results in vasodilatation with a tendency to desensitize (Zygmun 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 F₂α, such as potent contraction of the cat iris sphincter (Woodward et al., 2001), because only prostamide F₂α was found here to exert an appreciable, albeit very weak, TRPV1-mediated effect.

**Fig. 9.** Thin layer chromatography analysis of bimatoprost (0.5 mg) after 4-h 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 F₂α, the possible product of bimatoprost hydrolysis, was found to have an Rₚ similar to that of the prostaglandin F₂α, standard shown here.

**TABLE 5**

<table>
<thead>
<tr>
<th></th>
<th>F₂α</th>
<th>AEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ciliary</td>
<td>Iris</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected.
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 because, 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 h) 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 to be present, agrees with a previous study showing that prostamide E₂ is not hydrolyzed in either rat or human plasma (Kozak et al., 2001). Together with previous observations, our data on the refractoriness of prostamides to both enzymatic hydrolysis and cellular reuptake support the notion that these compounds are metabolically stable, inactivation products of AEA.

We also found that the prostamide analog 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-h 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-h) incubations (Maxey et al., 2002). The absence of prostamide F<sub>2</sub>α 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 (Re- sul 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 feedback mechanisms that inhibit FAAH-catalyzed AEA inactivation, as has been suggested for AEA oxidation by lipoygenases to products with FAAH inhibitory activity (van der Stelt et al., 2003). Our finding of weak activity by prostamide F<sub>2</sub>α 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 prostaglandin 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 nonacid congeners such as PG-glyceryl esters (Kozak et al., 2001) and PGF<sub>2</sub>α 1-alcohol and PGF<sub>2</sub>α 1-methoxy (Woodward et al., 2000).

Acknowledgments

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