Prostaglandin Ethanolamides (Prostamides): In Vitro Pharmacology and Metabolism


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Received October 16, 2003; accepted January 29, 2004

ABSTRACT

We investigated whether prostaglandin ethanolamides (prostamides) E2, F2α, and D2 exert some of their effects by 1) activating prostanoid receptors either per se or after conversion into the corresponding prostaglandins; 2) interacting with proteins for the inactivation of the endocannabinoid N-arachidonoyl ethanolamine (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 prostanoid 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-arachidonoyl ethanolamine (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-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-arachidonoyl glycine (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 prosta-

noid; 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-oxabicycl[2.2.1]hept-2-yl]-5-heptanoic acid.
15 times less potent than PGE2 in EP2 receptor-mediated relaxation of the guinea pig trachea (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 membranes; about 100-fold less potent than the free acid in functional 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 1-phenyl-derivative of prostamide F2α, known as bimatropost (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 F2α and D2 or of thromboxane and prostacyclin ethanalamides. 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). Prostamide E2 exhibits potency comparable with the corresponding prostaglandins in only a few assays of FP and EP2 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, because 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 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 synaptosomes or intact RBL-2H3 cells. Finally, we assessed the activity of all prostamides, in comparison with the corresponding prostaglandins, in two preparations expressing prostanoïd 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 molecular 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 sulprostone 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 [14C]AEA (specific activity 8 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 was synthesized in our laboratory as described previously (Bisogno et al., 1997).

#### Intracellular Calcium Concentration Assay

Human embryonic kidney (HEK)-293 cells overexpressing the human TRPV1 receptor were a kind gift from John Davis (GlaxoSmithKline, Harlow, UK). Cells were grown as monolayers in minimum essential 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 determined 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)...
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 methylster (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 the effect was determined by comparing it to the analogous effect observed with ionomycin (4 μM).

Cell Cultures

Rat basophilic leukemia (RBL-2H3) cells and mouse N18TG2 neuroblastoma cells were purchased from DSMZ (Braunschweig, 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 (Maurerelli et al., 1995), using membranes prepared from mouse neuroblastoma N18TG2 cells, incubated with the test compounds and [14C]AEA (8 μM; 20,000 cpm) in 50 mM Tris-HCl, pH 9, for 30 min at 37°C. [14C]Ethanolamine produced from [14C]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 [14C]AEA by intact rat basophilic leukemia (RBL-2H3) cells was studied using 4 μM (10,000 cpm) of [14C]AEA as described previously (Bisogno et al., 1997). Cells were incubated with [14C]AEA for 5 min at 37°C, in the presence or absence of the inhibitors (50 μM). Residual [14C]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 [14C]AEA disappeared from the medium of RBL-2H3 cells is found mostly (>90%) as unmetabolized [14C]AEA in the cell extract. Non-specific binding of [14C]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 with organic extracts containing residual [3H]prostamides from both the organic extracts and proteins (inactivated at 100°C for 30 min). The samples were 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 and F2, or with 75:25:1 chloroform/methanol/NH4OH for prostamide E2. The 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.

Bimatoprost (0.5 mg) 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 F2α (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°C or 4°C with prostamide E2 or F2α (5 μM; 30,000 cpm). Cellular and synaptosomal uptake of [14C]AEA (2.5 μM; 10,000 cpm) were determined 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 [14C]prostamides from both the incubation media, 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 7M). 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, 11.6 mM MgSO4, 25.0 mM NaHCO3, 11.7 mg glucose, and 0.001 mg ascorbic acid.

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 washout and return to baseline tension. The response to 10−8 M PGF2α 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 Alcohol 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 organ bath. The tissues were equilibrated for 1 h under 0.75-g tension, which was readjusted as the tissues relaxed. Single doses of histamine, 10−5 M then 2 to 3 × 10−5 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 to 3 ×
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 10⁻⁶ M to 10⁻⁴ M PGE₂, 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 PGE₂ 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 PGE₂ (10⁻⁴ 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 10⁻⁴ M PGE₂ 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 × 20-V pulses of 1-ms duration, each individual pulse separated by 100 ms. Electrical washout of each drug was allowed to elapse between testing of PGE₂ and reapplied at the conclusion of the experiment as a reference standard. One hour was allowed to elapse between testing of PGE₂ and reapplied at the conclusion of the experiment as a reference standard. Thirty to 45 min was allowed for tissue recovery after a cumulative manner. The response to 100 nM U-46619 was determined by an ability to cause aggregation (TP receptor activity). Fresh whole blood was obtained for any aggregatory activity. ADP (2 × 10⁻⁵ M) (final concentration) 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 10⁻⁴ M PGE₂ response.

Human Recombinant EP₁, EP₂, and TP Receptors: Binding Studies. Plasmids encoding the human EP₁, 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 EP₁ 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.

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 × 10⁻⁵ 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 × 10⁻⁵ 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 × 10⁻⁶ M ADP. Standard aggregatory responses to 2 × 10⁻⁶ M ADP alone were performed at the beginning and end of each experiment.

Human Recombinant EP₁, EP₂, EP₄, FP, and TP Receptors: Binding Studies. Plasmids encoding the human EP₁, EP₂, EP₄, 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 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-EBNA cells (Invitrogen) were grown in medium containing geneticin (G418) to maintain expression of the EBNA-1 protein. 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 prostanoïd receptor transfection, the cells were seeded into six-well plates at a density of 10⁵well⁻¹ the day before transfection. FuGENE 6 transfection agent (Roche Diagnostics, Indianapolis, IN) was diluted in OPTI-MEM (Invitrogen). The pCEP4 expression vector, containing cDNA for the required prostanoïd 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 pCEP₄ vector-prostanoïd receptor cDNA. This was followed by 2-d incubation. The cells were then transferred into 5 × 10⁶-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.

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 × 10⁻⁵ 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 × 10⁻⁵ 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 × 10⁻⁶ M ADP. Standard aggregatory responses to 2 × 10⁻⁶ M ADP alone were performed at the beginning and end of each experiment.

Human Recombinant EP₁, EP₂, EP₄, FP, and TP Receptors: Binding Studies. Plasmids encoding the human EP₁, EP₂, EP₄, and FP receptors were prepared by cloning the respective coding sequences into pcDNA3 vector (Invitrogen). COS-7 cells were transfected with pcDNA3 containing cDNA encoding the EP₁ 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 necessary to achieve a 40-ml volume in the centrifuge tubes. TME is composed of 50 mM Tris base, 10 mM MgCl₂, and 1 mM EDTA; pH 7.4 was achieved by adding 1 N HCl. The protein homogenate was centrifuged at 19,000 rpm for 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 protein. 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]PGE₂ (EP₁, EP₂, and EP₄ receptors) or 5 nM [³H]17-phenyl PGF₁₀ (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 flask, 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]PGE₂ at EP₁, EP₄, or 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]ISQ 29548, and nonspecific binding was determined with 10⁻⁵ M respective unlabeled prostaglandin.

**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 × 10⁵ 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, and EP₃ 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 Ggs cDNA containing a hemagglutinin (HA) epitope. The prostaglandin EP₃ receptor was cotransfected with chimeric Gq₁₁. Gq₁₁ and Gs chimeric 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 a specific antibody. In Western blot experiments, anti-HA-peroxidase and a mouse monoclonal antibody (clone 12 C5A) were used.

**Human Recombinant EP₁, IP, FP, 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 black-wall, 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 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 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 and E₂ were essentially equiactive 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-prostamide = 1827. The activity of PGE₂α-L-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 PGE₂α 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-L-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$_{2\alpha}$ and prostamide $F_{2\alpha}$ 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). EC$_{50}$ values (nanomolar) were obtained as follows: PGF$_{2\alpha}$ n = 2.8, prostamide $F_{2\alpha}$ n = 2000, PGD$_2$ n = 28, and prostamide $D_2$ n = 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 $F_{2\alpha}$ 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$_2$, 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 $F_{2\alpha}$ 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 $F_{2\alpha}$. PGD$_2$ and carbaprostacyclin served as positive controls and behaved as potent agonists at DP and
IP receptors, with respective EC\textsubscript{50} values of 40 and 5 nM (data not shown).

The activity of prostamide \textit{F}\textsubscript{2μ}/\textit{H9251} was also compared functionally in a range of cell and isolated tissue preparations. The data obtained are given in Table 3. Prostamide \textit{F}\textsubscript{2μ} was essentially inactive at human DP, IP, and TP receptors associated with platelets. In the isolated tissue and cell preparations, prostamide \textit{F}\textsubscript{2μ} exhibited modest activity and EC\textsubscript{50} values exceeded 10^{-6} M. Prostamide \textit{F}\textsubscript{2μ} was much less potent than the respective selective prostanoid receptor agonist for each preparation: guinea pig ileum (EP1), guinea pig vas deferens (EP\textsubscript{3}), endothelium-intact rabbit jugular vein (FP), and rat aorta (TP).

\textbf{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\textsubscript{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 \textit{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 \textit{F}\textsubscript{2μ} was capable of enhancing intracellular calcium with an EC\textsubscript{50} of 15.0 ± 2.4 \textmu M and a maximal effect that was 31 ± 2\% of the maximal effect observable with 4 \textmu M ionomycin (mean ± S.E.M., \textit{n} = 3) (Fig. 6). Prostamide \textit{D}\textsubscript{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 \textit{E}\textsubscript{2} was almost inactive in this test (Fig. 6). Bimatoprost did not elicit any response up to a 25 \textmu M concentration (data not shown). Under the same conditions, AEA and capsaicin exhibit EC\textsubscript{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 ± 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 [¹⁴C]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 [¹⁴C]AEA uptake by RBL-2H3 cells at any of the concentrations tested and up to 50 μM (Fig. 8).

**Metabolic Stability of Prostamides in Homogenates from Various Tissues.** The enzymatic hydrolysis of the prostamides was assessed by using the corresponding ³H-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, of³H-labeled corresponding prostaglandins were formed (Tables 4 and 5). Under the same conditions, 50–100% of [¹⁴C]AEA was hydrolyzed to [¹⁴C]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 ³H-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

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**Fig. 5.** Endothelium-intact rabbit jugular vein: a comparison of the vasorelaxant effects of PGD$_{2}$ and prostamide D$_{2}$ (a) and PGF$_{2\alpha}$ and prostamide F$_{2\alpha}$ (b). Values are mean ± S.E.M. n = 5 (a) and PGF$_{2\alpha}$ n = 6 (b).

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Prostanoid Receptor Subtype</th>
<th>Prostamide D$<em>{2}$ EC$</em>{50}$ (nM)</th>
<th>PGD$<em>{2}$ EC$</em>{50}$ (nM)</th>
<th>Prostamide E$<em>{2}$ EC$</em>{50}$ (nM)</th>
<th>PGE$<em>{2}$ EC$</em>{50}$ (nM)</th>
<th>Prostamide F$<em>{2\alpha}$ EC$</em>{50}$ (nM)</th>
<th>PGF$<em>{2\alpha}$ EC$</em>{50}$ (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>
<td>EP$_{1}$</td>
<td>NA</td>
<td>250</td>
<td>848</td>
<td>0.2</td>
<td>&gt;10,000</td>
<td>8.3</td>
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<tr>
<td>EP$_{2}$</td>
<td>NA</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>2.5</td>
<td>NA</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>EP$_{3}$</td>
<td>NA</td>
<td>120</td>
<td>123</td>
<td>0.3</td>
<td>NA</td>
<td>7.6</td>
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<tr>
<td>EP$_{4}$</td>
<td>NA</td>
<td>2,455</td>
<td>&gt;10,000</td>
<td>0.5</td>
<td>NA</td>
<td>6,457</td>
</tr>
<tr>
<td>FP$_{1}$</td>
<td>NA</td>
<td>13</td>
<td>NA</td>
<td>217</td>
<td>&gt;10,000</td>
<td>5</td>
</tr>
<tr>
<td>IP</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<td>NA</td>
<td>82</td>
<td>NA</td>
<td>49</td>
<td>NA</td>
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<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, \(^{14}\text{C}\)AEA 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 \(10 \mu\text{M}\) 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 \(N\)-arachidonoyl-glycine (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 CB1 and CB2 receptors (Berglund et al., 1999), support the
hypothesis that prostamides E2 and F2 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 E2 (Ross et al., 2002) or prostamide F2a (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 F2a, namely, the cat iris sphincter. The results were unexpected. The potencies of prostamides D2, E2, and F2a 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

**Fig. 7.** Effect of prostamides E2, F2a, and D2 and bimatoprost on FAAH activity. The inhibitory effect of these compounds (50 and 100 μM) were tested on the enzymatic hydrolysis of [14C]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 E2, F2a, and D2 and bimatoprost on anandamide cellular uptake. The lack of any significant inhibitory effect of these compounds (50 μM) on the uptake of [14C]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.

**TABLE 4**
Hydrolysis of prostamides into the corresponding prostaglandins by brain (prostamide E2, D2, and F2a), liver (prostamide F2a), and lung (prostamide F2a) 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 percentage of total radioactivity incubated (i.e., obtained from the same amounts of prostamides incubated with buffer). The percentage of hydrolysis of [14C]anandamide under the same conditions is also shown.

<table>
<thead>
<tr>
<th></th>
<th>E2</th>
<th>D2</th>
<th>F2a</th>
<th>AEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
<td>Brain</td>
<td>Brain</td>
<td>Brain</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>88.9 ± 1.7</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>93.5 ± 3.2</td>
</tr>
</tbody>
</table>

Brain: Brain; Liver: Liver; Lung: Lung

50 μM 100 μM

% control

0 20 40 60 80 100 120
TABLE 5
Hydrolysis of prostamide F2α, into the corresponding prostaglandins by cat iris sphincter and ciliary body homogenates

<table>
<thead>
<tr>
<th>pH</th>
<th>Ciliary Body</th>
<th>Iris</th>
<th>Ciliary Body</th>
<th>Iris</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>ND</td>
<td>23.5 ± 2.5</td>
<td>6.2 ± 0.1</td>
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<tr>
<td>9.0</td>
<td>ND</td>
<td>50.0 ± 5.8</td>
<td>46.7 ± 5.6</td>
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</tr>
</tbody>
</table>

ND, not detected.

sphincter seems distinctly sensitive to prostamides D2α, E2α, 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 amidates 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 the 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 PGD2α- and PGE2α-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 PGE2α was found to be about 200 times more potent than prostamide E2α in the rabbit jugular vein (Ross et al., 2002).

Prostamides D2α, E2α, 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 PGD2α, PGE2α, 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 (Zygmont et al., 1999). Furthermore, stimulation of TRPV1 receptors results in vasodilatation with a tendency to desensitize (Zygmont et al., 1999). These events are characteristic of bimatoprost-induced ocular surface hypoperemia (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.
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 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 unmethylated 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>2α</sub> 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 feedback mechanisms that inhibit FAAH-catalyzed AEA inactivation, as has been suggested for AEA oxidation by lipooxygenases to products with FAAH inhibitory activity (van der Stelt et al., 2002). Our finding of weak activity by prostamide F<sub>2α</sub> 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 nonacid congeners such as PG-glycerol esters (Kozak et al., 2001) and PGF<sub>2α</sub> 1-alcohol and PGF<sub>2α</sub> 1-methoxy (Woodward et al., 2000).

Acknowledgments

We thank Linda L. Johnson for preparation of the manuscript.

References

Bisogno T, De Petrocellis L, and Di Marzo V (1997) Biosynthesis of anandamide and other nonacid congeners such as PG-glycerol esters (Kozak et al., 2001) and PGF<sub>2α</sub> 1-alcohol and PGF<sub>2α</sub> 1-methoxy (Woodward et al., 2000).

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