Real-Time Intracellular Ca\(^{2+}\) Mobilization by Travoprost Acid, Bimatoprost, Unoprostone, and Other Analogs via Endogenous Mouse, Rat, and Cloned Human FP Prostaglandin Receptors

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Received August 13, 2002; accepted September 25, 2002

ABSTRACT

The ability of a number of prostaglandin \(F_{2\alpha}\) (PGF\(_{2\alpha}\)) analogs to mobilize intracellular Ca\(^{2+}\) \([\text{Ca}^{2+}]_i\) and to compete for \([\text{H}]\text{PGF}_{2\alpha}\) binding to prostaglandin \(F_{2\alpha}\) receptors (FP) was evaluated. Radioligand binding studies measuring displacement of \([\text{H}]\text{PGF}_{2\alpha}\) by a variety of FP prostaglandin analogs yielded the following rank order of affinities: travoprost acid (17-phenyl-trinor PGF\(_{2\alpha}\)) \(\gg\) unoprostone (13,14-dihydro-15-keto-20-ethyl PGF\(_{2\alpha}\)) \(\approx\) bimatoprost (17-phenyl-trinor PGF\(_{2\alpha}\) ethyl amide) \(\approx\) Lumigan (bimatoprost opthalmic solution). In FP functional studies, travoprost acid (EC\(_{50}\) = 17.5–37 nM, \(n = 13\)), bimatoprost acid (EC\(_{50}\) = 23.3–49.0 nM, \(n = 6–12\)), unoprostone (EC\(_{50}\) = 306–1270 nM, \(n = 4–8\)), bimatoprost (EC\(_{50}\) = 3070–3940 nM, \(n = 4–9\)), and Lumigan (EC\(_{50}\) = 1470–3190 nM, \(n = 5–9\)) concentration dependently stimulated \([\text{Ca}^{2+}]_i\) mobilization via the rat (A7r5 cells), mouse (3T3 cells), and cloned human ocular FP prostaglandin receptors. The rank order of potency of these compounds at the FP receptor of the three species was similar and in good agreement with the determined binding affinities. The agonist effects of these compounds were concentration dependently blocked by the FP receptor-selective antagonist, AL-8810 (11β-fluoro-15-epi-15-indanyl-tetranor PGF\(_{2\alpha}\)) \(K_i = 0.6–1.3\ \mu\text{M}\). These studies have demonstrated that bimatoprost, unoprostone, and bimatoprost acid possess direct agonist activities at the rat, mouse, and human FP prostaglandin receptor and that travoprost acid is the most potent of the synthetic FP prostaglandin analogs tested.

Prostaglandin \(F_{2\alpha}\) receptor (FP receptor) agonists are potent and efficacious agents that have been shown to reduce elevated intraocular pressure (IOP) in humans and nonhuman primates (Wang et al., 1990; Bito, 1997). A number of prostaglandin \(F_{2\alpha}\) (PGF\(_{2\alpha}\)) analogs are marketed for the treatment of ocular hypertension, a major risk factor for glaucoma. The ocular anti-hypertensive drugs travoprost (TRAVATAN) and latanoprost (Xalatan) are well characterized as FP receptor agonists (Bhattacherjee et al., 2001). This has been the subject of some debate as to whether these drugs exhibit any biological activities independent of any known prostaglandin receptors (Bhattacherjee et al., 2001). Thus, we sought to fully characterize the pharmacological activities of bimatoprost and additional PGF\(_{2\alpha}\) analogs through FP receptor binding using similarity to PGF\(_{2\alpha}\) bimatoprost is claimed not to be a prodrug of bimatoprost acid but to exert its actions via a postulated and characterized “prostamide” receptor (Woodward et al., 2001). This has been the subject of some debate as recent evidence suggests that human and bovine corneal tissues convert bimatoprost to its free acid and that this hydrolys product may account for the observed IOP reduction (Maxey et al., 2002).

Unoprostone isopropyl ester is the prodrug of unoprostone. Unoprostone is a 13,14-dihydro-15-keto analog of PGF\(_{2\alpha}\) having a two-carbon extension of the \(\alpha\) side chain. (Taniguchi et al., 1996). Unoprostone (and its isopropyl ester) has recently been reported to have no observed activity at FP or other known prostaglandin receptors (Bhattacherjee et al., 2001; Woodward et al., 2001) despite being PGF\(_{2\alpha}\) analogs.

Bimatoprost is the ethyl amide derivative of 17-phenyl trinor PGF\(_{2\alpha}\), a potent prostaglandin FP receptor agonist (Woodward et al., 2001). Despite this inherent structural ABBREVIATIONS: FP, prostaglandin \(F_{2\alpha}\) receptor; IOP, intraocular pressure; PGF\(_{2\alpha}\), prostaglandin \(F_{2\alpha}\); HEK, human embryonic kidney; AL-8810, 11β-fluoro-15-epi-15-indanyl-tetranor PGF\(_{2\alpha}\); [\text{Ca}^{2+}]_i, intracellular calcium concentration; FLIPR, fluorometric imaging plate reader; BCLM, bovine corpus luteum membrane; PI, phosphatidylinositol.
Materials and Methods

Cell Culture. A7r5 rat vascular smooth muscle cells and Swiss albino mouse 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose, 110 mg/ml sodium pyruvate, pyridoxine hydrochloride, and GlutaMax I, and supplemented with 10 μg/ml gentamicin sulfate and 10% fetal bovine serum. HEK-293 cells expressing the full-length cloned human ciliary body FP receptor (HEK-FP cells) were grown in the above media supplemented with 0.4 mg/ml geneticin. Cells were propagated at 5- to 7-day intervals by treatment with 0.05% trypsin-0.55 mM EDTA.

Materials. A7r5 smooth muscle cells derived from embryonic rat aorta and Swiss albino mouse 3T3 fibroblasts (CRL 1444) were purchased from the American Type Culture Collection (Manassas, VA). HEK-293 cells expressing the cloned human ciliary body FP receptor were obtained from Dr. G. Fitzgerald (University of Pennsylvania, Philadelphia, PA) and cultured as previously described (Kunapuli et al., 1997). Cell culture media, antibiotics, and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT) heat-inactivated at 56°C for 30 min and stored at −20°C. [3H]Prostaglandin F2α (150–175 Ci/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). Bimatoprost free acid (17-phenyl trinor PGF2α), bimatoprost (17-phenyl trinor PGF2α ethyl amide), unoprostone, unoprostone isopropyl ester, and other standard prostanoids were from Cayman Chemical (Ann Arbor, MI). Lumigan (0.03% bimatoprost ophthalmic solution) was obtained from Allergan, Inc. (Irvine, CA). Travoprost free acid and AL-8810 were synthesized in the Medicinal Chemistry Department of Alcon Research, Ltd. Fluorometric imaging plate reader (FLIPR) and calcium assay kit dyes were from Molecular Devices Corp. (Sunnyvale, CA).

[3H]Prostaglandin F2α Binding Studies. Competitive prostaglandin FP receptor binding was performed using washed bovine corpus luteum membrane preparations (20 mg/ml in Krebs buffer, pH 7.4) as previously described (Sharif et al., 1998). Briefly, washed bovine corpus luteum membrane (BCLM) homogenates were incubated with [3H]Prostaglandin F2α (1 nM) and increasing concentrations of the test compound for 2 h at 23°C. Nonspecific binding was defined using 1 μM unlabeled PGF2α or (-)-fluprostenol. Assays were terminated by rapid vacuum filtration using Whatman GF/B glass fiber filters previously soaked in 0.3% polyethylenimine. Receptor-bound radioactivity was determined by liquid scintillation spectrometry at 50% efficiency.

Intracellular Ca2+ Mobilization Studies. [Ca2+]i mobilization studies were performed using a FLIPR as previously described (Sullivan et al., 1999; Jerman et al., 2000; Sharif et al., 2001). Briefly, A7r5, 3T3, or HEK-FP cells were seeded into black-walled, clear-bottom 96-well plates at a cell density of 25,000 to 50,000 cells/well in normal media 48 h before experiment. Cells in normal media were then loaded with a calcium kit assay dye in FLIPR buffer (Hanks’ balanced salt solution [Ca2+]i = 1.3 mM) buffered with 20 mM Hepes, pH 7.4, containing 2.5 mM procenebic) and incubated at 23°C for 1 h. Test compounds were diluted in 10% dimethyl sulfoxide/10% ethanol and then in FLIPR buffer and evaluated in 5- and 10-point concentration-response formats. Agonist-stimulated intracellular calcium mobilization was measured using a FLIPR 1 system (Molecular Devices Inc.) monitoring changes in cellular fluorescence (λex = 488 nm, λem = 540 nm) before and after addition of various agonists. Calibration of the FLIPR 1 instrument was performed as per the manufacturer’s standard procedures. For antagonist studies, dye-loaded cells were incubated with varying concentrations of the FP

Table 1: FP receptor affinities of selected prostaglandin analogs competing for specific [3H]PGF2α binding to BCLMs

<table>
<thead>
<tr>
<th>Compound</th>
<th>FP Receptor Binding Affinity (Kd ± S.E.M.)</th>
<th>Hill Coefficient</th>
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<tr>
<td>Bimatoprost acid ([+]-fluprostenol)</td>
<td>49.9 ± 3.3</td>
<td>0.8 ± 0.02</td>
</tr>
<tr>
<td>Bimatoprost acid (17-phenyl trinor PGF2α)</td>
<td>85.0 ± 14.0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Unoprostone (acid) (UP-021)</td>
<td>3580 ± 687</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Lumigan (amide; bimatoprost ophthalmic solution; Allergan, Inc.)</td>
<td>3426 ± 1225</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>Bimatoprost (amide; Cayman Chemical Co.)</td>
<td>9862 ± 4738</td>
<td>0.8 ± 0.02</td>
</tr>
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Fig. 1. Competition curves for prostaglandin analogs displacing specific [3H]PGF2α binding to BCLM membranes. Data are mean ± S.E.M. from n = 3 to 7 independent experiments.

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Fig. 2. Intracellular Ca\textsuperscript{2+} mobilization in rat A7r5 cells. A7r5 cells were loaded with a Ca\textsuperscript{2+}-sensitive dye and exposed to test compounds, and the change in fluorescence was measured over 180 s. The representative fluorescence traces show concentration-dependent mobilization of [Ca\textsuperscript{2+}]\textsubscript{i} in response to travoprost acid (A), bimatoprost acid (B), bimatoprost (C), Lumigan (D), unoprostone (free acid) (E), and PGF\textsubscript{2\alpha} (F).
receptor-selective antagonist, AL-8810, for 15 min before addition of agonists.

Results

[^3H]PGF2α Binding. Competition binding of prostanoids to the FP receptor was performed using the natural ligand[^3H]PGF2α, and native FP receptors expressed in BCLM preparations. In a typical binding experiment, specific binding of[^3H]PGF2α, (1 nM) to BCLM represented >80% of total binding observed. In concentration-inhibition binding studies, all prostaglandin analogs tested inhibited the specific binding of[^3H]PGF2α to BCLM in a concentration-dependent manner (Fig. 1). Travoprost acid [(+)-fluprostenol], a potent and highly selective FP receptor agonist, and bimatoprost acid (17-phenyl- trinor PGF2α) strongly displaced[^3H]PGF2α binding to FP receptors. Bimatoprost (17-phenyl- trinor PGF2α ethyl amide), Lumigan (0.03% bimatoprost ophthalmic solution), and unoprostone, the free acid form of the prodrug unoprostone isopropyl ester, completely inhibited[^3H]PGF2α binding to FP receptors in BCLM (Fig. 1 and Table 1). These data are in good agreement with similar prostaglandin FP receptor binding studies conducted with BCLM homogenates utilizing the FP receptor-selective radioligand[^3H]travoprost acid (Sharif et al., 2002a).

Intracellular Calcium Mobilization. All prostanoid compounds tested, including bimatoprost and unoprostone, elicited a robust and rapid (<5 s post drug addition) increase in relative fluorescence corresponding to increased [Ca2+]i, monitored in real time with the FLIPR via the rat, mouse, and human FP receptor. Representative traces of [Ca2+]i mobilization mediated by PG analogs in rat A7r5 cells are presented in Fig. 2. Interestingly, the real-time fluorescence traces for the tested prostaglandin analogs differ in their respective kinetic profiles. Travoprost acid, bimatoprost acid, and the FP natural ligand PGF2α elicited a rapid spike in [Ca2+]i, followed by a prolonged plateau that failed to return to baseline within the time course of the experiment (180 s) (Fig. 2, panels A, B, and F). In contrast, bimatoprost, Lumigan, and unoprostone free acid elicited an immediate, robust spike in [Ca2+]i that rapidly decayed back to baseline levels (<180 s) (Fig. 2, panels C, D, and E). As expected, the FP receptor natural ligand, PGF2α, exhibited a concentration-dependent mobilization of calcium in A7r5 cells with a representative EC50 of 15.2 nM (Fig. 2, panel F). The observed [Ca2+]i mobilization response induced by PG analogs was concentration-dependent (Fig. 3) and the resulting agonist potencies (EC50) of the compounds are shown in Table 2. The rank order of FP receptor agonist potency obtained (travoprost acid ≥ bimatoprost acid ≥ unoprostone ≥ bimatoprost/Lumigan ) was comparable across the three species studied (Table 2).

In addition, the FP receptor-selective antagonist, AL-8810, inhibited the [Ca2+]i mobilization response mediated by travoprost acid, bimatoprost acid, bimatoprost/Lumigan, and unoprostone in the A7r5 cells. In the presence of AL-8810, there was a concentration-dependent reduction in the observed Ca2+ response to prostanoid agonists (Fig. 4). Pre-treatment with 100 μM AL-8810 completely abolished the
Fig. 3. Concentration-dependent mobilization of \([\text{Ca}^{2+}]_i\) in A7r5 cells by various prostaglandin analogs: travoprost acid (■), bimatoprost acid (□), bimatoprost (▲), Lumigan (▼), and unoprostone (free acid) (●). Responses induced by the different compounds were represented as a percentage of the maximal response generated by 10 \mu M (±)-fluprostenol. Data are mean ± S.E.M. from \(n = 4\) to 13 experiments.

Table 2

<table>
<thead>
<tr>
<th>Compounds</th>
<th>FP Receptor Potency EC_{50} ± S.E.M. (nM)</th>
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<tbody>
<tr>
<td></td>
<td>Swiss 3T3 Cells (mouse)</td>
</tr>
<tr>
<td>Travoprost acid ([+]-fluprostenol)</td>
<td>37.3 ± 4.5</td>
</tr>
<tr>
<td>Bimatoprost acid (17-phenyl trinor PGF_2α)</td>
<td>49.0 ± 5.2</td>
</tr>
<tr>
<td>Unoprostone (acid) (UF-021)</td>
<td>407 ± 83</td>
</tr>
<tr>
<td>Lumigan (amide; bimatoprost ophthalmic solution; Allergan, Inc.)</td>
<td>2670 ± 449</td>
</tr>
<tr>
<td>Bimatoprost (amide; Cayman Chemical Co.)</td>
<td>3120 ± 729</td>
</tr>
</tbody>
</table>
Fig. 4. Inhibition of $[Ca^{2+}]_i$ mobilization by the FP receptor-selective antagonist, AL-8810. Representative fluorescence traces show the diminished $[Ca^{2+}]_i$ mobilization in response to a fixed agonist concentration in the presence of increasing concentrations of AL-8810: A, travoprost acid (TA), 100 nM; B, bimatoprost acid, 100 nM; C, bimatoprost, 3 μM; D, Lumigan, 3 μM; and E, unoprostone (free acid), 1 μM.
cellular processing, such as hydrolysis or conversion to other compounds via metabolism.

Swiss 3T3 mouse fibroblasts have previously been shown to express functional FP receptors associated with prostanoid-induced Ca\(^{2+}\) signals (Woodward and Lawrence, 1994; Griffin et al., 1997). Our studies here extend previous work showing similar functional agonist activity for bimatoprost and unoprostone at the mouse FP receptor in these cells (Table 2 and Sharif et al., 2001). Furthermore, utilizing HEK-293 cells expressing the cloned human ocular FP receptor from ciliary body (Kunapuli et al., 1997), travoprost acid, bimatoprost acid, bimatoprost, and unoprostone also rapidly mobilized \([Ca^{2+}]_i\) and exhibited a rank order potency comparable across species.

The FP receptor antagonist, AL-8810, is considered to be of great utility as a pharmacologic and diagnostic tool for investigating FP receptor-mediated biological responses (Sharif and Griffin, 2002). In fact, AL-8810 has previously been shown to selectively inhibit fluprostenol-mediated PI turnover in A7r5 cells (Griffin et al., 1999) and to displace \(^3\)HPGF\(_{2\alpha}\), binding to BCLM (Sharif et al., 2000). Based on previous Schild analyses using PI turnover, AL-8810 behaves as a competitive inhibitor at the endogenous FP receptors of A7r5 and 3T3 cells (Griffin et al., 1999). In contrast, another analog of PGF\(_{2\alpha}\), 11-deoxy-16-fluoro PGF\(_{2\alpha}\) (AL-3138), exhibited properties consistent with a noncompetitive mechanism of antagonism in these same cell and assay systems (Sharif et al., 2000).

Several recent studies have shown that fluprostenol and travoprost acid [(\(\pm\))-fluprostenol] are more selective agonists at the FP receptor than the natural FP ligand, PGF\(_{2\alpha}\), which displays considerable activity at prostaglandin E\(_2\) receptors (Sharif et al., 1998; Abramovitz et al., 2000; Hellberg et al., 2001; Ungrin et al., 2001). Further validation of the specificity of AL-8810 toward the FP receptor is shown here by the effective abolition of travoprost acid-induced \([Ca^{2+}]_i\) mobilization in A7r5 cells. Indeed, AL-8810 effectively inhibited the agonist-induced mobilization of calcium observed with all prostanoids tested in a concentration-dependent manner. The antagonist potency determined for AL-8810 inhibition of \([Ca^{2+}]_i\) mobilization in A7r5 cells using travoprost acid, bimatoprost acid, bimatoprost/Lumigan or unoprostone as the agonist was quite similar (\(K_I = 0.6–1.3\) M) in this study. These values are in very good agreement with inhibitory potencies of AL-8810 obtained in PI turnover at the cloned human ciliary body FP receptor with several of these same agonists (\(K_I = 1.0–2.1\) M) (Sharif et al., 2002b). The complete inhibition of calcium mobilization by AL-8810 confirms the FP receptor nature of the responses elicited by bimatoprost and unoprostone and suggests that the observed agonist activity of these prostaglandin analogs is entirely attributable to FP receptor activation.

Although travoprost and latanoprost are known to mediate their pharmacological actions via the FP prostaglandin receptor (Stjernschantz et al., 1995; Hellberg et al., 2001), the biological actions of bimatoprost and unoprostone have been the subject of some debate. In contrast to our findings presented here, Woodward et al. (2001) did not observe significant binding of bimatoprost at any of the classical prostaglandin receptors, including the FP receptor. The reason for this discrepancy is unclear;
however, it is difficult to reconcile these contradictory findings since the radiolabeled ligand and tissue used by Woodward et al. (2001) were not specified and the functional testing was undefined. In our hands, bimatoprost readily bound to the FP receptor and displaced \(^{3}H\)travoprost acid (Sharif et al., 2001) and \(^{3}H\)PG\textsubscript{F} \(_{2a}\) (this study).

Bhattacherjee et al. (2001) found no specific binding of unoprostone isopropyl ester or unoprostone using \(^{3}H\)unoprostone isopropyl in bovine corpus luteum membranes and competing with unlabeled unoprostone isopropyl. However, given our findings that unoprostone isopropyl and unoprostone free acid have micromolar binding affinities, we feel it would be difficult to detect appreciable specific binding using nanomolar concentrations of this weak radioligand. Bhattacherjee et al. (2001) also reported that unoprostone failed to mobilize \([Ca^{2+}]_i\), in primary human ciliary muscle cells. We have found that \([Ca^{2+}]_i\) mobilization in response to FP prostaglandins in human ciliary muscle cells can be quite weak and donor dependent, perhaps suggesting that low receptor expression and/or poor signal coupling in these primary cultures may contribute to the lack of response observed with weak FP agonists.

In conclusion, we have clearly demonstrated that sources of bimatoprost (from Cayman Chemical Co. and as Lumigan from Allergan, Inc.) as well as unoprostone 1) specifically bind to FP receptors in BCLM; 2) directly activate endogenous FP receptors of rat A7r5 cells and mouse 3T3 fibroblasts, and also stimulate functional responses via the cloned human ocular FP receptors; and 3) can have their actions inhibited by a selective, competitive FP receptor antagonist.

Acknowledgments

We thank Drs. Tom Dean and Mark Hellberg for valuable discussions and critical review of the manuscript.

References


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