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The Hydrolysis of Bimatoprost in Corneal Tissue Generates a Potent Prostanoid FP Receptor Agonist

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Abstract. Using human and bovine corneal tissue, we investigated the in vitro metabolism of bimatoprost (17-phenyl-18,19,20-trinor-prostaglandin $F_{2\alpha}$ ethyl amide, Lumigan (Allergan, Inc, Irvine, CA). Enzymatic amidase activity, which converts bimatoprost to the corresponding prostaglandin carboxylic acid, was found to be present in corneal tissue from both species. Using HPLC and mass spectrometry for analyses, conversion of bimatoprost to 17-phenyl-18,19,20-trinor prostaglandin $F_{2\alpha}$ continued for at least 24 hours after excision of the cornea, with a conversion rate of approximately 25 μg/24 hours. This hydrolysis product is identical to the free acid of latanoprost with the exception of a double, rather than a single, bond at the carbon 13-14 position. Assuming that this conversion also occurs in vivo at a similar rate, this hydrolysis product may account for the reduction of intraocular pressure occurring in patients treated with bimatoprost. (**Surv Ophthalmol 47(Suppl 1)**:S34–S40, 2002. © 2002 by Elsevier Science Inc. All rights reserved.)

Key words. bimatoprost • cornea • FP receptor • glaucoma • hydrolysis • prostaglandin

Prostaglandins in the eye were believed to be ocular hypertensives and proinflammatory agents prior to the pioneering work of Bito and Camras in 1977.[3,6] Subsequent studies by these authors and others demonstrated that by selecting an appropriate F-series prostaglandin analog and rendering it a lipophilic prodrug through derivitization of the C-1 carboxyl group, potent and effective ocular hypotensive drugs were created.[15,17] The earliest of such drugs to be approved for the treatment of glaucoma were Xalatan[5] (Pharmacia Corp, Peapack, NJ) and Rescula[18] (Ciba Vision, Atlanta, GA), followed very recently by Travatan[16] (Alcon Pharmaceuticals, Ft Worth, TX) and Lumigan[7] (Fig. 1). These agents have revolutionized the treatment of glaucoma in humans, and they are poised to become the first line of drug therapy for this prevalent, sight-threatening disorder. A key common feature of all these agents is the ability of the parent prostaglandin carboxylic acid to stimulate prostaglandin FP receptors.[1,2] Although the exact mechanism of the ocular hypotensive action of prostaglandins is still subject to debate, FP receptor activation has generally been considered to be a pivotal step in the process.

Unlike the other drugs mentioned above, bimatoprost acquires its lipophilic character through derivitization of C-1 as an ethyl amide, rather than the more common isopropyl ester. It has further been claimed that bimatoprost does not undergo hydrolysis to the parent free acid in the eye, and that its mechanism of action does not include the prostaglandin FP receptor.[20] We sought in this study to determine whether prostaglandin C-1 amides are hydrolyzed to the parent free acid in human and bovine corneal tissue.

Methods

MATERIALS

Bimatoprost, travoprost, latanoprost, unoprostone isopropyl ester, their corresponding free acids, and

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PII S0039-6257(02)00323-5

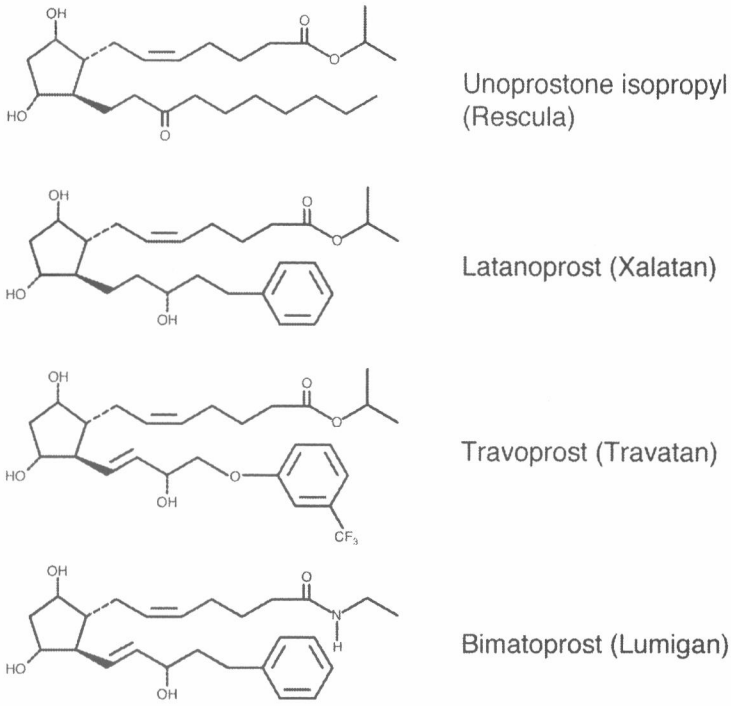


Fig. 1. Prostaglandin analogs approved for glaucoma treatment.

all other prostaglandin standards were synthesized in the Organic Chemistry Department at Cayman Chemical. Human corneas were from the Michigan Eye Bank and were kindly provided by Dr. Bruce Cohan. Bovine corneas were from healthy animals and were collected from the abattoir at the time of death. All other reagents were of research reagent grade and were from Sigma or similar commercial sources.

INCUBATION/PRODUCT ANALYSES

Corneas (human or bovine) were excised shortly after death and immediately refrigerated to 0–4°C; corneas were utilized 6 to 48 hours later. The enzymatic activity of corneas older than 48 hours was absent or reduced, so experiments were run only on fresh tissue. The corneas were placed into glass test tubes containing 5 ml of 20 mM phosphate buffered saline ([PBS], pH 7.4). Control test tubes contained only 5 ml of PBS (no cornea). 17-phenyl-trinor $PGF_{2\alpha}$ ethylamide (bimatoprost), $PGF_{2\alpha}$ isopropyl ester, or another prostaglandin analog (250 μg in 100 μl ethanol for human corneas, or 400 μg in 200 μl ethanol for bovine corneas) was added to one test tube containing corneal tissue and one control test tube containing only PBS. The test tubes were incubated with gentle shaking at 37°C. Aliquots (2 ml) were taken after 6 and 23 hours for testing. The reaction mixture was acidified to pH 3 with 5% $KHSO_4$, saturated with NaCl, and extracted three times with 80% ethyl acetate:hexane. The solvent was evaporated under nitrogen and then brought up in 200 μl of 80% ethyl acetate:hexane. The samples were analyzed by high-performance liquid chromatography (HPLC) on a reverse-phase C_{18} column (Beckman; Ultrasphere, 5 μ, 4.6 × 250 mm) using methanol:water:acetic acid (70:30:0.1v/v) as the mobile phase at a flow rate of 1 ml/min. The eluent was monitored at 210 nm. Analysis by thin layer chromatography (TLC) was performed by spotting 5 μl of the organic phase extract on silica gel G-60 plates (Analtech) which were eluted with 95:5:1 ethyl acetate/methanol/acetic acid and developed by charring under sulfuric acid spray.

MASS SPECTROMETRY

Mass spectrometry was carried out on a Finnigan Mat LCQ mass spectrometer. The human cornea hydrolyzed bimatoprost product was collected during HPLC and analyzed without derivitization. A mass range of m/z 105–500 was scanned at the rate of 3 sec/scan using a negative ion electrospray LC/MS.

IMMUNOBLOTTING

Immunoblotting was carried out according to the standards of Towbin et al.[19] Briefly, the resolved proteins from the acrylamide gel (12%) were transferred to a nitrocellulose membrane and blocked with 5% nonfat dry milk in 25 mM Tris-HCl, pH 8.0, containing 137 mM NaCl. The membrane was incubated with a specific antiserum to rat fatty acid amide hydrolase raised in rabbits that was kindly provided by Dr. Cecilia Hillard at the Medical Col-

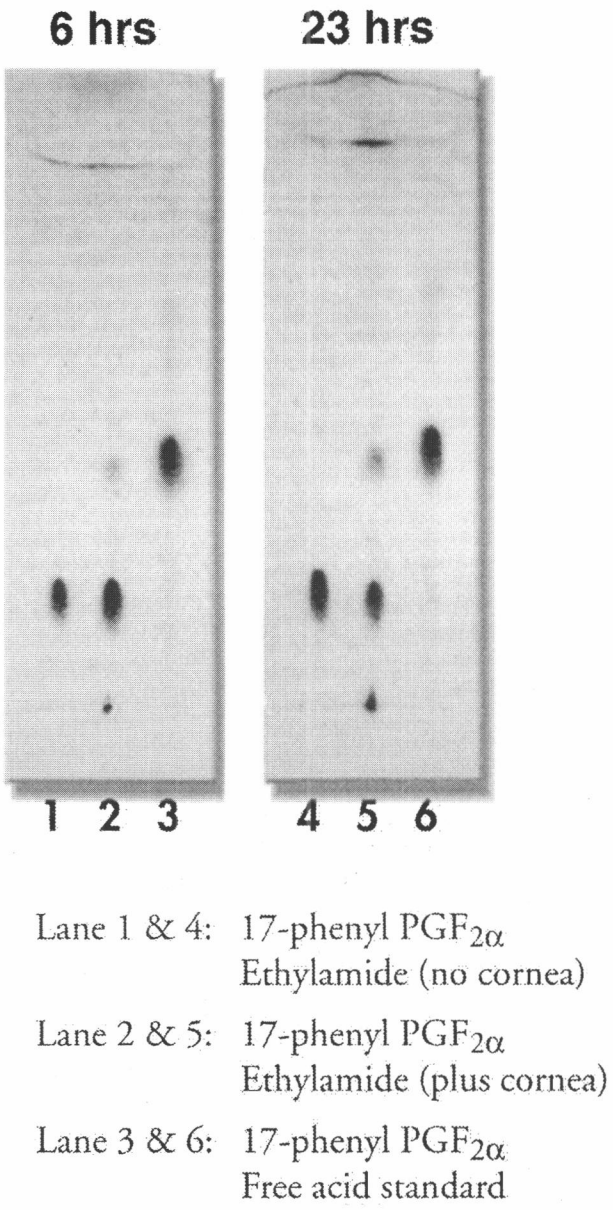


Fig. 2. Thin layer chromatography (silica gel G-60) analysis of the ethyl acetate extracts of phosphate buffered saline containing 400 μg/ml of bimatoprost and a single bovine cornea at 6- and 23-hour time points.

lege of Wisconsin. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) was used as the second antibody for detection.

Results

BOVINE CORNEA HYDROLYSIS

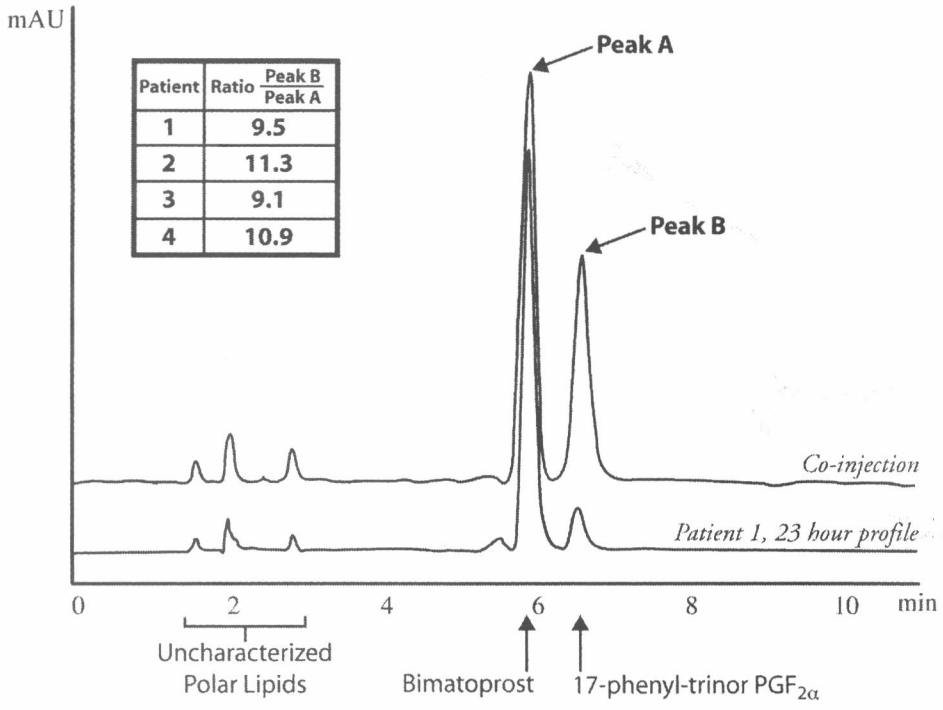
The products of the hydrolysis of bimatoprost using bovine cornea were first analyzed by TLC and are shown in Fig. 2. Within 6 hours of starting the incubation, and more noticeably at 23 hours, a new product which co-migrated with the 17-phenyl-trinor Prostaglandin $F_{2\alpha}$ free acid standard was evident. These results were then extended by repeating the experiment with analysis of the product by HPLC, which was at least five times more sensitive with respect to the detection threshold for prostaglandins (2–3 μg for TLC vs. 0.3–0.5 μg for HPLC). The HPLC analysis confirmed the presence of a new prostanoid in 23-hour hydrolysis experiments, which co-eluted with authentic 17-phenyl-trinor prostaglandin $F_{2\alpha}$ free acid standard. By integrating the area under the curve of the respective peaks, we estimated the new product to represent approximately 8% of the original amide prodrug, or 32 μg of free acid active metabolite (average of three experiments).

HUMAN CORNEA HYDROLYSIS

The products of the hydrolysis of bimatoprost using human corneas were analyzed by HPLC and are shown in Fig. 3. The hydrolysis was qualitatively similar, but slightly more complete, with 10% (25 μg) of the active free acid metabolite present at 23 hours (average of 4 experiments). In order to confirm the identity of the new metabolite, which co-eluted with 17-phenyl-trinor prostaglandin $F_{2\alpha}$ free acid, we collected the peak from the HPLC analysis and analyzed it by mass spectrometry. The resulting spectrum, shown in Fig. 4, is identical to 17-phenyl-trinor prostaglandin $F_{2\alpha}$ free acid and confirms that this compound is the only major metabolite formed during incubation of bimatoprost with human cornea.

STRUCTURE ACTIVITY RELATIONSHIP

In order to investigate the structural requirements of the amidase/esterase activity in the cornea, we prepared various combinations of prostanoid core structure and lipophilic group. The results are summarized in Table 1. Since we determined using bimatoprost that the hydrolysis was easily reproducible, with a variance of less than 10%, we performed only a single hydrolysis experiment with each of the new C-1 derivatives. Although the numbers presented might be refined by further repetition, we are comfortable that the general trend is clear. We found that enzymatic activity was present which was relatively insensitive to changes in the prostaglandin parent compound, in that it accepted phenyl-substituted and N-alkyl substituted lower side chain analogs with equal avidity. Isopropyl and methyl esters were hydrolized well, but the monosubstituted N-ethyl amide (as in bimatoprost) was hydrolized at only 10% of the rate for isopropyl esters. Adding a second N-ethyl group (17-phenyl trinor $PGF_{2\alpha}$-N,N′-diethyl amide) abolished any detectable substrate activity and led only to the recovery of unmetabolized starting compound.



Patient	Ratio Peak B / Peak A
1	9.5
2	11.3
3	9.1
4	10.9

Fig. 3. High-performance liquid chromatography chromatogram of a sample of bimatoprost taken after incubation for 23 hours in buffer with a human cornea (lower trace). The upper trace is the same sample after the co-injection of authentic 17-phenyl-trinor-$PGF_{2\alpha}$. The numbers next to each patient (box) represent the percentage of bimatoprost (Peak A) which has been converted to free acid (Peak B).

Discussion

The hydrolysis of prostaglandin isopropyl esters in the eye has been reported in numerous studies.[5,11] However, the enzyme or enzymes responsible for this hydrolysis have not been carefully studied or characterized. Woodward presented data showing no detectable hydrolysis of bimatoprost in homogenized iris ciliary body over a 2.5-hour period.[20] However, the analytical method Woodward used was insensitive and would not have detected small amounts of hydrolysis; further, the study used ciliary body rather than cornea, which is the tissue thought to activate the glaucoma prodrugs and the tissue where the drug is first absorbed. Termination of the Woodward study at 2.5 hours was certainly premature, since bimatoprost is a once-daily drug with well documented ocular hypotensive effects 24 hours after dosing.

In this study, we have demonstrated that prostaglandin C-1 N-ethyl amides, like isopropyl esters, are also hydrolized in the eye, in this case by corneal tissue. The human cornea contains sufficient amidase activity to convert more than 25 μg of bimatoprost to the corresponding free acid in a 24-hour period. Since the normal dose of bimatoprost is 7.5 μg per day, this raises the possibility that a significant amount of the free acid, 17-phenyl-trinor $PGF_{2\alpha}$, is produced in treated eyes. If as little as 0.01% of this dose accumulated in the aqueous humor as the free acid, the concentration (about 2 nM) would be sufficient to provide significant FP receptor occupancy.[1] The IOP-lowering effect of bimatoprost could therefore be entirely due to FP receptor activation. This provides a more rational explanation of the IOP-lowering effect of bimatoprost than that which has been offered to date, since the alternate theory postulates the existence of an undiscovered receptor for which no clone, sequence, tissue distribution, or pharmacological profile is known.[7] Analysis of the aqueous humor of bimatoprost-treated patients for the free acid metabolite will be necessary to further support this hypothesis.

The ability of the primate eye to convert both ester and amide prodrugs to their corresponding free acid metabolites has been well documented.[4,13] However, the identity of the specific enzyme(s) responsible, and a detailed structure-activity relationship (SAR) has not been published. Table 1 shows the results of a very limited SAR evaluation of this hydrolytic activity. Both methyl and isopropyl esters of $PGF_{2\alpha}$ are converted almost quantitatively to the free acids by the cornea within 24 hours. The enzyme(s) responsible for this conversion seem relatively insensitive to the structure of the PGF analog, in that extended omega chains, (unoprostone isoproplyl ester) and phenyl-substituted (travoprost and latanoprost) analogs are converted with about the same efficiency as the parent prostaglandin, $PGF_{2\alpha}$. The amide analogs, in contrast, show a clear impairment of hydrolysis compared to the ester prodrugs. Hydrolysis of the amides is also markedly inhibited by N-alkyl groups. As demonstrated in Table 1, a single ethyl group on

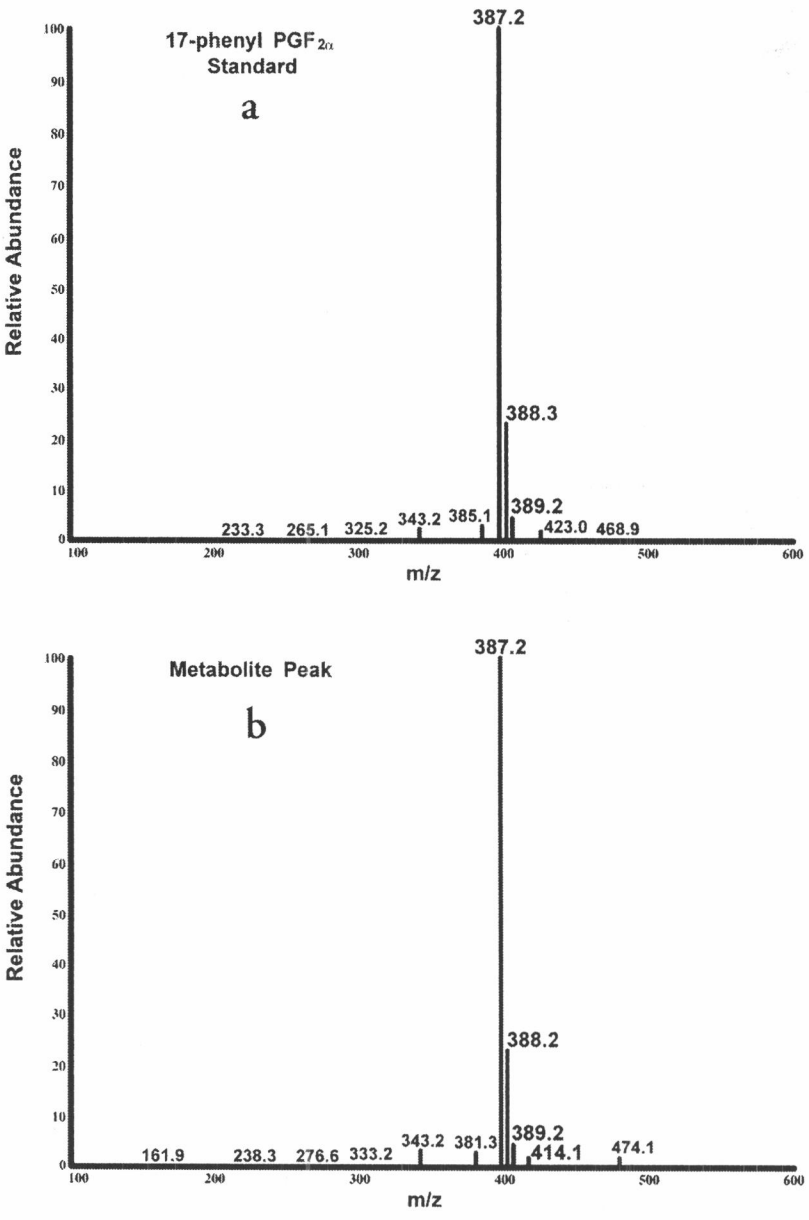


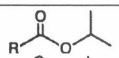
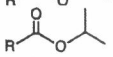
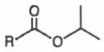
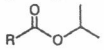
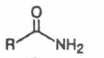
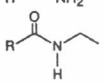
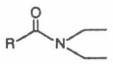
Fig. 4. Negative ion electrospray LC/MS of Peak B collected from high-performance liquid chromatography chromatogram in Fig. 3 (*b*) and the same mass spectrum obtained using an authentic sample of 17-phenyl-trinor-$PGF_{2\alpha}$ (*a*).

the amide nitrogen decreases the already inefficient hydrolysis by 50%, and a second N-ethyl ligand completely abolishes it.

Fatty Acid Amide Hydrolase (FAAH) is a recently discovered enzyme with both amidase and esterase activity.[8,10] FAAH functions biologically to metabolize and inactivate the two primary endocannabinoids, arachidonoyl ethanolamide (AEA, anandamide) and 2-arachidonoyl glycerol (2-AG).[9,14] Cravatt et al have shown that FAAH is relatively non-discriminating as to the size and degree of unsaturation in the fatty acid component of potential amide substrates, which supports our findings in Table 1.[8] The cannabinoid receptors CB1 and CB2 on which these endocannabinoids act are present in the eye, making it seem plausible that the deactivating enzyme FAAH would also be present.[12] Interestingly, one of these compounds is an amide (AEA) and the other (2-AG) is an ester. It is possible that FAAH is the single enzyme present in ocular tissues which is responsible for the activation of all prostaglandin prodrugs. A recent report of FAAH expression in rat ocular tissues found expression in several retinal cell types; however, these authors did not investigate the cornea.[21] We performed immunoblots with fresh, homogenized human cornea using a peptide antibody raised against the rat FAAH sequence, and transfected HEK cells expressing the human FAAH protein as a positive control, shown in Fig. 5. A clear band of immunoreactivity with the expected Mr of 57 kDa has been tentatively identified as human FAAH.[12] Current studies in our lab seek to confirm

TABLE 1

Hydrolysis of Representative Prostaglandin Esters and Amides by Human Cornea After An Overnight Incubation With 250 μg of the Prodrug

Compound	C-1 Derivative	% Hydrolysis
$PGF_{2\alpha}$ isopropyl ester (n = 2)		87%
Latanoprost (n = 1)		79%
Unoprostone isopropyl ester (n = 1)		71%
Travoprost (n = 1)		78%
17-phenyl-trinor-$PGF_{2\alpha}$-amide (n = 2)		20%
Bimatoprost (n = 4)		10%
17-phenyl-trinor-$PGF_{2\alpha}$ N,N′-diethyl amide (n = 2)		0%

$PGF_{2\alpha}$ methyl ester served as the positive control, and is defined as 100%. Concentrations of the prodrug and free acid were measured by high-performance liquid chromatography.

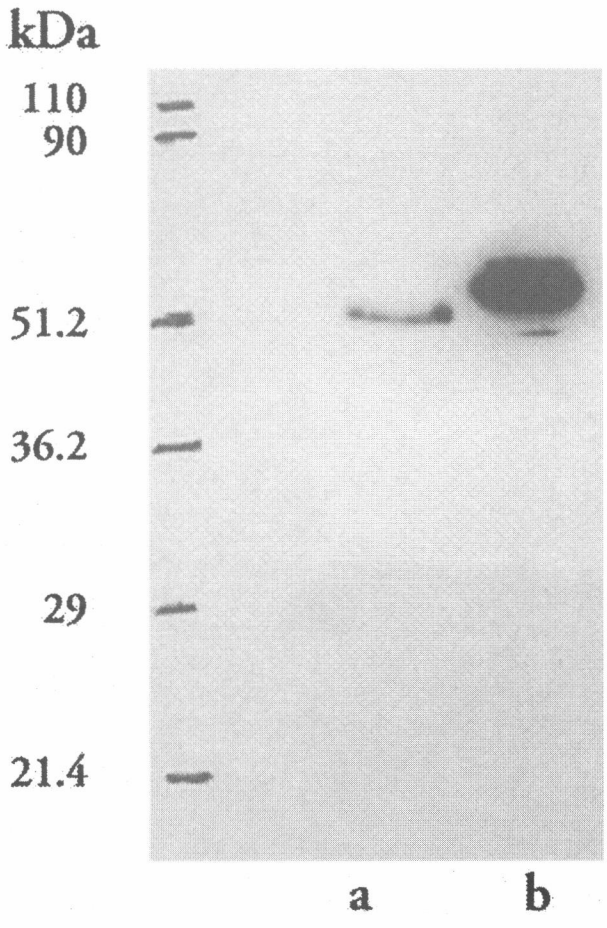


Fig. 5. Immunoblot of human cornea. Western blot of human corneal homogenate (*a*) and human recombinant fatty acid amide hydrolase as postive control (*b*), using the polyclonal anti-rat fatty acid amide hydrolase antibody.[18]

whether FAAH is present in the human cornea, and whether it is responsible for the hydrolysis of bimatoprost and other prostaglandin prodrugs.

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Cayman Chemical Company provided 100% of the funding for this article. Kirk M. Maxey, MD, is a paid consultant for Pharmacia, the manufacturer of Xalatan. The authors are employees of Cayman Chemical, which makes and sells bimatoprost, latanoprost, unoprostone, and other PGs as research reagents.

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