Effect of the Enzyme Inhibitor, Phenylmethylsulfonyl Fluoride, on the IOP Profiles of Topical Anandamides

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PURPOSE. Earlier studies have suggested that the intraocular pressure (IOP) effects of topical arachidonylethanolamidamide (AEA) are mediated through its fatty acid metabolite, rather than through AEA, per se. The purpose of this study was to investigate whether the topical anandamides AEA and arachidonoylpropionitrileamide (APN) decrease IOP when their enzymatic degradation is prevented by phenylmethylsulfonyl fluoride (PMSF) and whether the neuronal cannabinoid (CB1) receptor mediates the IOP responses of an undegraded AEA, through the use of its specific antagonist SR141716A.

METHODS. AEA or APN were each formulated in aqueous 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) solutions and administered unilaterally to the rabbit eye (dose, 62.5 μg per rabbit). To prevent the degradation of AEA or APN, the rabbits were pretreated with a subcutaneous (SC) PMSF injection (0.22–22 mg/kg) 30 minutes before eye drop instillation. To determine whether the neuronal cannabinoid (CB1) receptor mediates the hypotensive IOP effects of undegraded AEA, the rabbits were pretreated with simultaneous SC injections of a CB1 receptor antagonist SR141716A (1.2–2.1 mg/kg) and PMSF (2.2 mg/kg) before the ocularly applied AEA.

RESULTS. In the absence of PMSF, the IOP profiles of AEA and APN showed a biphasic ocular effect—that is, an initial increase of IOP followed by IOP hypotension in the treated eye. In the presence of PMSF (2.2 mg/kg for AEA and 22 mg/kg for APN), IOP profiles showed immediate IOP reduction in the treated eye. SR141716A antagonized the IOP reduction caused by the undegraded AEA.

CONCLUSIONS. These results indicate that the apparently undegraded AEA and APN decrease IOP in normotensive rabbits. AEA-induced IOP reduction in the presence of PMSF is probably mediated through a CB1 receptor. (Invest Ophthalmol Vis Sci. 2002;43:393–397)

It was originally observed that subjects who smoked marijuana (Cannabis sativa L.) had reduced intraocular pressure (IOP).1 This resulted in numerous studies exploring cannabinoiids as possible antiglaucoma drugs.2 The endogenous cannabinoid arachidonylethanolamide (anandamide, AEA) was first isolated in porcine brain3 and was shown to bind to the neuronal CB1 receptor and subsequently to the peripheral cannabinoid receptor.4–6 AEA mimics many of the pharmacologic effects of Δ9-tetrahydrocannabinol,7 the major psychoactive constituent of Cannabis. Other endogenous cannabinoids, including 2-arachidonoylglycerol, have been found in various tissues,8–10 suggesting that this class of compounds may form an entire family of chemical mediators in the body.

AEA has a very short duration of action in vivo. Its inactivation occurs by facilitated transport through the cell membrane,11–12 followed by an enzymatic hydrolysis13 to arachidonic acid and ethanolamine by fatty acid hydrolyase (FAAH)15–17 (Fig. 1). Thus formed, the arachidonic acid can serve as a precursor for prostaglandin synthesis.18,19 The presence of FAAH has been reported in a variety of porcine ocular tissues,20 and the bovine retina has been shown to enzymatically release AEA.21 Discovery of CB1 receptor mRNA in the rat eye12 and, subsequently, CB1 receptor protein in the human eye,23 particularly in the ciliary body, provides physioanatomical support for a possible role of cannabinoid receptors in controlling IOP.

Topical AEA has been shown to affect rabbit IOP in a dose-dependent manner.24–25 AEA evokes various degrees of a biphasic ocular effect—that is, initial increase followed by IOP hypotension. The mechanism by which AEA produces its IOP effects is not yet known. Pate et al.26 have implied a role for prostanoids and the cyclooxygenase (COX) system with AEA and other α-unsubstituted anandamides, because their IOP effects can be eliminated with an indomethacin pretreatment. α-Substituted anandamides and synthetic cannabinoids, such as CP55940 and WIN55212-2, seem to act through CB1 receptors.27–29

In this study, we investigated whether undegraded AEA and its synthetic nitrile analogue arachidonoylpropionitrileamide (APN) decrease IOP in normotensive rabbits. Enzymatic degradation of AEA and APN was prevented by phenylmethylsulfonyl fluoride (PMSF). PMSF is a nonselective serine protease inhibitor that blocks the activity of FAAH and prevents the hydrolysis of anandamides.14,16 We also investigated whether a CB1 receptor mediates the IOP responses of the undegraded AEA, through the application of a specific CB1 receptor antagonist SR141716A.

MATERIALS AND METHODS

Chemicals

AEA was purchased from Organix Inc. (Woburn, MA) and APN was obtained from Deva Biotech, Inc. (Hatboro, PA). 2-Hydroxypropyl-β-cyclodextrin HP-β-CD (Encapsin; molecular weight, 1297.4; degree of molar substitution, 0.4) was purchased from Janssen Biotech (Olen, Belgium), and SR141716A was kindly donated by Sanofi Research (Montpellier, France). PMSF was obtained from Aldrich Chemie (Steinheim, Germany) and dissolved in glycerol formal (Sigma Chemical Co., St. Louis, MO). Isotonic 0.9% sodium chloride solution, at pH 4.5 to 7.0, was purchased from Orion Pharma (Espoo, Finland).

Animals

The experimental animals used in this study were normotensive Dutch belt rabbits of either gender (2.3–4.0 kg, n = 5–6). The rabbits were housed singly in cages under standard laboratory conditions: 12-hour dark–12-hour light cycle, 20.0 ± 0.5°C and 55% to 75% relative humid-
An ethanol solution of AEA or APN was evaporated under a stream of nitrogen and redissolved in aqueous 10% HP-β-CD (AEA) or 15% HP-β-CD (APN) solutions. The vehicle controls contained 10% or 20% HP-β-CD. The pH of eye drop solutions was adjusted to 7.4 with sodium hydroxide, and the solutions were made isotonic with sodium chloride. Drug concentrations of the AEA or APN solutions was 2.5 mg/mL (0.25%). Drug concentrations of the eye drop solutions were analyzed by high-pressure liquid chromatography (HPLC).

**Drug Analysis**

The analytical HPLC system for determination of drug concentrations in the eye drops was a commercial system (Merck Hitachi; Hitachi, Tokyo, Japan), including a pump (model L-7100), a diode array detector (model L-7455; 200–400 nm), and a programmable autosampler (model L-7250). An end-capped reversed-phase column (Purosphere RP-18; 125 × 4 mm, 5 μm, Merck kGaA, Darmstadt, Germany) was used for separations. A mobile-phase mixture of acetonitrile and 0.02 M phosphate buffer solution (pH 7.0) was used at a flow rate of 1.2 mL/min. The proportion of acetonitrile in the mobile phase was increased linearly from 60% to 90% over 15 minutes, maintained for 5 minutes, and returned to the initial condition over the course of 6 minutes. Quantitation of the compounds was made from measurements of their peak areas in relation to standards chromatographed under the same conditions.

**Preparation and Administration of the PMSF, Indomethacin, and SR141716A Solutions**

PMSF was dissolved in glycerol formal to concentrations of 3, 30, and 150 mg/mL. Depending on the test procedure, each rabbit received approximately 0.22, 2.2, and 22.0 mg/kg PMSF in a subcutaneous (SC) injection, 30 minutes before application of ocular AEA or APN, because of its low solubility in the ophthalmic vehicle. Indomethacin was dissolved in an aqueous 20% HP-β-CD solution to a concentration of 5 mg/mL. The solution pH was adjusted to 7.4 with aqueous sodium hydroxide, and the solution was made isotonic with sodium chloride. Each rabbit in the indomethacin study received 12.5 mg (3.1–5.4 mg/kg) of indomethacin by SC injection approximately 30 minutes before the ocular AEA treatment.

An SR141716A solution (0.3 mg/mL) was prepared by dissolving the compound in a 42% HP-β-CD solution of phosphate buffer at pH 4.2. Each rabbit in the antagonism study received 4.8 mg (1.2–2.1 mg/kg) SR141716A by SC injection, 30 minutes before ocular AEA treatment. SC pretreatments with a 0.9% sodium chloride solution were used as control treatments. The single-dose levels of cannabinoid agonists and the antagonist used were selected from previous studies.24,27–28

**IOP Measurements**

To perform each test, a rabbit was placed in a plastic restraining box located in a quiet room. A single drop (25 μL) of the test solution or vehicle was instilled unilaterally into the left eye, on the upper corneoscleral limbus. The contralateral eye was left untreated. During instillation, the upper eyelid was pulled slightly away from the globe. IOP was measured using a pneumotonometer (Digilab Modular One; Bio-Rad, Cambridge, MA). Before each measurement, 1 or 2 drops of oxybuprocaine (0.06%) was applied to the cornea to eliminate discomfort. The upper and lower eyelids were then gently retracted, and the sensor was brought into contact with the center of the cornea. For each determination, at least two readings were taken from each treated (ipsilateral) and untreated (contralateral) eye, and the mean of these readings was used. IOP of the rabbits was measured at 2, 1, and 0 hours before, and at 0.5, 1, 2, 3, 4, and 5 hours after, eye drop administration. IOP at the time of eye drop administration (0 hour) was used as a baseline value. All studies were set up using a masked and randomized crossover design. At least 72 hours of washout time was allowed for each rabbit between doses.

**Analysis of the Data**

Results are presented as a change in IOP (in millimeters of mercury) mean ± SE (n = 5–6). A one-factor analysis of variance (ANOVA) for repeated measurements was used to test the statistical significance of differences between groups. Significance in the differences of the means was tested using the Fisher’s protected least significant difference (PLSD) method at the 95% confidence level. Significance in the differences of the means between the two treatments was tested using a two-tailed paired Student’s t-test evaluated at the 95% confidence level.

**RESULTS**

**Treated Eyes**

Ocually applied AEA (62.5 μg) caused an initial increase of IOP in the treated eye followed by a statistically significant IOP reduction, when compared with a 10% HP-β-CD control solution. The maximal IOP reduction (~4.6 ± 1.4 mm Hg) occurred 3 hours after treatment (Fig. 2A). A smaller initial increase of IOP in the treated eye was also observed after ocular administration of APN (62.5 μg), a synthetic AEA analogue. A maximal decrease of IOP (~3.4 ± 1.1 mm Hg) was observed 2 hours after administration of this compound (Table 1).

To prevent AEA or APN catabolism in the eye, PMSF was administered by SC injection 30 minutes before eye drop instillation. IOP profiles of apparently undegraded AEA and APN exhibited immediate ocular hypotension after PMSF pretreatment (PMSF dose 2.2 mg/kg for AEA and 22 mg/kg for APN). The maximal IOP reduction (~3.9 ± 1.7 mm Hg) occurred 2 hours after AEA administration in the presence of PMSF (Fig. 2A). Table 1 shows that the maximal IOP reduction (~3.8 ± 1.3 mm Hg) was obtained 3 hours after ocular administration of APN with PMSF. PMSF pretreatment at a dose of 0.22 mg/kg for AEA and 2.2 mg/kg for APN did not eliminate the initial in-
TABLE 1. IOP Changes in the Treated Eyes of Rabbits after Administration of Test Compounds

<table>
<thead>
<tr>
<th>Ocular Treatment</th>
<th>Subcutaneous Pretreatment</th>
<th>Time after Eye Drop Application (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>APN</td>
<td>0.9% NaCl</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>APN</td>
<td>PMSF 2.2 mg/kg</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>APN</td>
<td>PMSF 22.0 mg/kg</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20% HP-β-CD</td>
<td>0.9% NaCl</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>AEA</td>
<td>0.9% NaCl</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>AEA</td>
<td>PMSF 0.22 mg/kg</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>AEA</td>
<td>PMSF 2.2 mg/kg</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>10% HP-β-CD</td>
<td>0.9% NaCl</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>10% HP-β-CD</td>
<td>PMSF 0.22 mg/kg</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>10% HP-β-CD</td>
<td>PMSF 2.2 mg/kg</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>10% HP-β-CD</td>
<td>PMSF 22 mg/kg</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>AEA</td>
<td>PMSF 2.2 mg/kg + SR141716A</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>10% HP-β-CD</td>
<td>PMSF 2.2 mg/kg + SR141716A</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Data are mean mm Hg ± SE (n = 5–6). Dose was 62.5 μg/25 μL.
*Statistically significant compared with vehicle treatment (10% HP-β-CD or 20% HP-β-CD with 0.9% NaCl; P < 0.05; ANOVA, Fisher’s PLSD).
IOP increase was observed in treated and untreated eyes. Presence of the FAAH inhibitor, PMSF, hypotensive IOP effects were not reversed because the COX inhibitor (indomethacin) pretreatment had no influence on the IOP reduction effects of AEA in the presence of PMSF (Table 2). These results suggest that the ocular hypotension seen after ocular administration of AEA or APN, in the presence of the FAAH inhibitor PMSF, is due to unmetabolized AEA or APN. However, it should be noted that PMSF is also a potent inhibitor of cholinesterases and other serine proteases. 

A specific CB1 cannabinoid receptor antagonist, SR141716A, was used to examine whether the IOP reduction induced by AEA in the presence of PMSF is mediated through CB1 receptors. In earlier studies, SR141716A has failed to antagonize the ocular effects of AEA. In this study, in the presence of the FAAH inhibitor, PMSF, hypotensive IOP effects of AEA were inhibited by the CB1 receptor antagonist, and an IOP increase was observed in treated and untreated eyes. Similar bilateral hypertension was observed when the CB1 receptor antagonist and PMSF were administered in conjunction with the ocular vehicle. This observation is consistent with earlier studies and supports the hypothesis that the CB1 receptor may be involved with the physiological control of IOP. SR141716A may increase IOP by acting as an antagonist for the endogenous cannabinoid receptor agonist, blocking its tonic regulatory effects on IOP. SR141716A has also been reported to act as an inverse agonist under certain test conditions.

In conclusion, this study reports for the first time that ocularly administered AEA, an endogenous ligand of the CB1 receptor, and its synthetic analogue APN reduced IOP without the initial IOP hypertensive phase if their enzymatic degradation was prevented by the enzyme inhibitor PMSF. The IOP reduction induced by AEA in the presence of PMSF was mediated through a CB1 receptor, suggesting that the endocannabinoid system may have a physiological role in the regulation of ocular tension.

Acknowledgments

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References


Table 2. Effect of COX Inhibitor Indomethacin on the IOP Profile of AEA in the Presence of PMSF in Treated Eyes of Rabbits

<table>
<thead>
<tr>
<th>Ocular Treatment</th>
<th>Subcutaneous Pretreatments</th>
<th>Time after Eye Drop Application (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>AEA</td>
<td>PMSF (22 mg/kg), 0.9% NaCl</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>AEA</td>
<td>PMSF (22 mg/kg), Indomethacin (12.5 mg/rabbit)</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Data are mean mm Hg ± SE (n = 6). Dose of AEA was 62.5 μg. No statistical significances were observed between the treatments (P > 0.18, two-tailed paired Student’s t-test).

FIGURE 3. IOP changes (mean ± SE, n = 5) in treated (A) and untreated (B) eyes of normotensive pigmented rabbits after unilateral ocular administration of AEA (62.5 μg) and SC administration of PMSF (2.2 mg/kg) with or without SC SR141716A (4.8 mg) pretreatment. *Significantly different from AEA administered with PMSF (P < 0.05, ANOVA, Fisher’s LSD).