Effects of Topical Anandamide-Transport Inhibitors, AM404 and Olvanil, on Intraocular Pressure in Normotensive Rabbits

Krista Laine,[1,5] Tomi Järvinen,[1] Juha Savinainen,[2] Jarmo T. Laitinen,[2] David W. Pate,[1,3] and Kristiina Järvinen[4]

Received November 27, 2000; accepted December 8, 2000

Purpose. To evaluate the effects of topically applied anandamide transport inhibitors, AM404 and olvanil, on the intraocular pressure (IOP) of normotensive rabbits. To determine if the ocular hypotension induced by topical anandamide (AEA) can be potentiated by co-administered AM404.

Methods. Test compounds, in either hydroxypropyl-β-cyclodextrin (HP-β-CD) or propylene glycol, were administered unilaterally onto rabbit eyes. To determine if AM404 affects the IOP-profile of AEA, AM404 was administered ocularly 15 minutes before topical AEA. Phenylmethylsulfonyl fluoride (PMSF) (24 mg/kg, s.c.) was given 30 min before AEA to prevent its catabolism. IOPs of the treated and untreated eyes were measured. The cannabinoid agonist activities of AM404 and olvanil were studied by using [^{35}S]GTPγS autoradiography.

Results. Topical AM404 (62.5 μg), in HP-β-CD vehicle, decreased IOP significantly in treated eyes. AM404 (62.5 μg) induced a significant IOP increase without subsequent decrease when given in propylene glycol vehicle. Olvanil (312.5 μg) caused a significant IOP reduction without provoking an initial hypertensive phase. These compounds did not significantly affect the IOP of untreated eyes. Co-administered AM404 (125 μg in HP-β-CD) had no significant effect on the IOP profile of AEA (62.5 μg).

Conclusions. Ocular administration of AM404 or olvanil decreased IOP in rabbits, although AM404 can provoke an initial ocular hypertension and did not potentiate the IOP responses induced by exogenous AEA.

KEY WORDS: anandamide (AEA); N-(4-hydroxyphenyl)-arachidonyl amide (AM404); (N-vanillyl)-9-oleamide (olvanil); anandamide transport inhibitors; intraocular pressure (IOP); [^{35}S]guanosine 5′-(λ-thio)triphosphate; autoradiography; rabbits.

INTRODUCTION

Ocular (1) and systemic (2) administration of cannabinoids, including Δ^9-tetrahydrocannabinol (Δ^9-THC), lowers intraocular pressure (IOP). Arachidonyl ethanolamide (anandamide, AEA) (3) and 2-arachidonyl glycerol (2-AG) (4) are putative endogenous neuronal cannabinoid receptor (CB1) ligands. They and their various chemical derivatives mimic many of the pharmacological actions of cannabinoids, including IOP effects (5–7) which can be blocked by the highly specific CB1 receptor antagonist SR141716A (8). The discovery of a CB1 receptor in the rat eye (9) and subsequently in the eyes of other animal species (10), including humans (11), further supports a physiological role for endogenous cannabinoid signalling system in the eye.

AEA inactivates rapidly *in vivo*. The inactivation occurs by carrier-mediated transport into cells (12) followed by enzymatic hydrolysis (13) via intracellular fatty acid amide hydrolase (FAAH). Accumulation of AEA fulfils the requirements for facilitated transport (14), that is, it is rapid ($t_{1/2}$ = 2.6 min), saturable, temperature-dependent and possesses strict substrate selectivity. Cellular uptake is reported to be competitively and effectively inhibited by an aromatic anandamide analog, N-(4-hydroxyphenyl)-arachidonyl amide (AM404) (15), and by a vanilloid receptor agonist, (N-vanillyl)-9-oleamide (olvanil) (16).

It has been reported earlier that co-administered AM404 significantly enhances and prolongs AEA-induced analgesia in mice (15). Calignano et al. (17) have shown an enhanced vasodepressor response and prolonged duration of action to AEA after co-administration with AM404 in anesthesized guinea pigs. In both studies, AM404 had little or no effect when administered without AEA.

The aim of the present study was to investigate the effect of topically applied AM404 or olvanil on the IOP of normotensive rabbits. We also evaluated the effects of AM404 on the IOP profile induced by exogenous AEA.

MATERIALS AND METHODS

Chemicals

N-(4-hydroxyphenyl)-arachidonyl amide (AM404) was purchased from Cayman Chemical (Ann Arbor, MI, USA) or from Deva Biotech (Hatboro, PA, USA). Anandamide (AEA) was purchased from Deva Biotech (Hatboro, PA, USA) and (N-vanillyl)-9-oleamide (olvanil) from Tocris Cookson Ltd. (Bristol, UK). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD, Encapsin, mw = 1297.4, degree of molar substitution = 0.4) was obtained from Janssen Biotech (Olen, Belgium) and propylene glycol from Oriola OY (Espoo, Finland). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Aldrich Chemie (Steinheim, Germany), isotonic 0.9% sodium chloride solution from Orion Pharma (Espoo, Finland) and glycerol formal from Sigma Chemical Company (St. Louis, MO, USA).

For the [^{35}S]GTPγS autoradiography, sources of chemicals were as follows: 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) from RBI (Natick, MA, USA); BSA (fatty acid free),

[1] Department of Pharmaceutical Chemistry, University of Kuopio, Finland.
[2] Department of Physiology, University of Kuopio, Finland.
[3] HortaPharm B.V., Amsterdam, The Netherlands.
[4] Department of Pharmaceutics, University of Kuopio, Finland.
[5] To whom correspondence should be addressed. (e-mail: krista.laine@uku.fi)

ABBREVIATIONS: AEA, anandamide (arachidonyl ethanolamide); AM404, N-(4-hydroxyphenyl)arachidonyl amide; BSA, bovine serum albumin; CB1, neuronal cannabinoid receptor; CP-55,940, (−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol]; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DTT, dithiothreitol; FAAH, fatty acid amide hydrolase; GTPγS, guanosine 5pri-[λ-thio]triphosphate; HP-β-CD, hydroxypropyl-β-cyclodextrin; IOP, intraocular pressure; PMSF, phenylmethylsulfonyl fluoride; *R*-methAEA, *R*-1-methanandamide; SR 141716A, [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride].

0724-8741/01/0400-0494$19.50/0

GDP, GTPγS, dithiothreitol (DTT) and PMSF from Sigma (St. Louis, MO, USA). [^{35}S]GTPγS (initial specific activity 1250 Ci/mmol) was purchased from DuPont NEN. The stock solution was diluted 1:50 in 10 mM Tris-HCl (pH 7.4) containing 10 mM DTT, and apportioned into single-use aliquots stored at −80°C. CP-55,940 {(−)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclo-hexyl]-phenol}was a gift from Pfizer Inc. (Groton, CT, USA) and R-1-methanandamide was synthesized by the method of Devane et al. (3). SR 141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride], the selective CB1 receptor antagonist (18), was provided by Sanofi Research (Montepellier, France). All other chemicals were of the highest purity available.

Preparation of Eyedrops and PMSF Solution

AM404 or AEA were dissolved in ethanol for transfer. The ethanol was then evaporated under a nitrogen steam. AM404 was redissolved in an aqueous 25% HP-β-CD solution or in propylene glycol. AEA was redissolved in an aqueous 10% HP-β-CD solution. The pH of the aqueous eyedrop solutions was adjusted to 7.4 with sodium hydroxide and the solutions were made isotonic with sodium chloride. Final drug concentrations of the AM404 and AEA solutions were 2.5 mg/ml. Olvanil was dissolved in propylene glycol to concentrations of 2.5 mg/ml and 12.5 mg/ml. Either a 25% HP-β-CD solution or propylene glycol was used as a control vehicle in the AM404 studies, or propylene glycol was the control vehicle in the olvanil studies. Drug concentrations were analysed by high-pressure liquid chromatography (HPLC) as described below. PMSF was dissolved in glycerol formal to a concentration of 150 mg/ml.

Determination of Drug Concentrations

A gradient HPLC method was developed for the determination of drug concentrations in the eyedrop solutions. The HPLC system consisted of a Merck Hitachi (Hitachi Ltd., Tokyo, Japan) L-7100 pump, Merck Hitachi L-7000 interface module, Merck Hitachi diode array detector (200–400 nm, set to 211 nm) and a Merck Hitachi L-7250 programmable autosampler. A Purospher RP-18 endcapped reverse-phase column (125 mm × 4 mm, 5 μm) protected with a Purospher RP-18 endcapped guard column (40 mm × 4 mm, 5 μm) was used for separations. The chromatographic conditions were as follows. The mobile phase consisted of acetonitrile and a 0.02 M phosphate buffer solution of pH 7.0, run at a flow rate of 1.2 ml/min. The proportion of acetonitrile in the mobile phase was increased from 60% to 95% via a linear ramp of 15 min, then maintained on a 5 min plateau, and subsequently returned to initial conditions over the course of 8 min.

Intraocular Pressure (IOP) Measurements

Experimental animals used in this study were normotensive Dutch Belted rabbits of either gender (2.3–4.0 kg, n = 5–6). The rabbits were housed singly in cages under standard laboratory conditions: 12 h dark / 12 h light cycle, 20.0 ± 0.5°C and 55–75% relative humidity. Rabbits were given water and food *ad libitum* except during the tests. This research adhered to the "Principles of Laboratory Animal Care."

To perform each test, the rabbits were placed in plastic restraining boxes located in quiet room. A drop (25 μl or 50 μl) of test solution was instilled unilaterally into each of their left eyes, on the upper corneoscleral limbus. During the instillation, the upper eyelid was pulled slightly away from the globe. IOP was measured using a BioRad (Cambridge, MA) Digilab Modular One Pneumatonometer. Before each measurement, one or two drops of topical anesthetic (0.06% oxybuprocaine) were applied to the cornea before tonometry to eliminate discomfort. The upper and lower eyelids were then gently retracted, and the applanation sensor was brought into contact with the center of cornea. For every determination, at least two readings were taken from each treated (ipsilateral) and untreated (contralateral) eye and the mean of these readings was used. IOP was measured at 2, 1, and 0 h before, and at 0.5, 1, 2, 3, 4 and 5 h after, eyedrop administration. IOP at the time of eyedrop administration (0 h) was used as the baseline value. Baseline IOPs ranged between 14.4–32.6 mm Hg. The studies were set up using a masked and randomized crossover design. At least 72 h of wash-out time was allowed for each rabbit between dosings.

When the effects of AM404 and olvanil on IOP were studied, olvanil (62.5 μg or 312.5 μg, 25 μl) and AM404 (62.5 μg, 25 μl) topical eyedrop solutions were ophthalmically administered. However, for the study of how AM404 (125 μg, 50 μl, 15 min prior to AEA) affects the IOP profile induced by AEA (62.5 μg in HP-β-CD vehicle, 25 μl), PMSF (approximately 24 mg/kg) was given as a subcutaneous injection 30 min prior to the topical AEA ophthalmic dose in order to inhibit AEA hydrolysis. PMSF was not given ocularly because its low aqueous solubility hindered eyedrop formulation. Control treatments for the above-mentioned experiments were as follows. PMSF (24 mg/kg, s.c.) was administered 30 min prior to topical application of the ocular HP-β-CD vehicle. An ophthalmic 0.9% NaCl solution (50 μl) was administered 15 min prior to the HP-β-CD vehicle in substitution for the AM404 treatment.

[^{35}S]GTPγS Autoradiography

[^{35}S]GTPγS autoradiography was conducted using optimized assay conditions wherein the prominent signal due to tonic adenosine A_1 receptor activity has been eliminated (19,20). Twenty-μm-thick brain sections (two coronal and two sagittal sections per slide, each from an individual animal) from 4-week-old male Wistar rats were used. The assay consisted of a pre-incubation for 20 min at 20°C in buffer A (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$), followed by GDP loading for 1 h at 20°C in buffer A additionally containing 2 mM GDP and 1 μM DPCPX. For [^{35}S]GTPγS binding, sections were incubated for 90 min at 20°C in buffer A with added 0.3–0.5 nM [^{35}S]GTPγS, 2 mM GDP, 1 μM DPCPX, 1 mM DTT and cannabinoid ligands in concentrations indicated by Fig. 5 dissolved in 0.1 or 2% (w/v) fatty acid-free BSA. Non-specific binding was determined in the presence of 10 μM [^{35}S]GTPγS. The brain sections were washed twice at 0°C for 5 min each time in a washing buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4), rinsed in ice-cold deionized water for 30 sec, air dried and apposed to Hyperfilm™-βmax (Amersham) for 5 days together with autoradiographic [^{14}C] microscale standards (Amersham). Films were developed in Kodak D-19 developer for 4 min at 4°C.

Data Analysis

The results are presented as a change in the IOP (mm Hg) mean, ± S.E. (standard error) of the mean. Significance in the differences of the means between the two treatments was tested by using a 2-tailed paired Student's *t*-test evaluated at the 95% confidence level. The statistical differences between multiple groups were tested by using a one-factor analysis of variance (ANOVA) for repeated measures. The significance in the differences of the means was evaluated by using the Fisher's Protected Least Significant Difference (PLSD) method at the 95% confidence level.

RESULTS

Treated Eye

Chemical structures of the experimental compounds are presented in Fig. 1. Topical ocular administration of AM404 (62.5 μg, 25 μl) in a HP-β-CD vehicle markedly reduced IOP compared to the vehicle treatment (Fig. 2A). The IOP reduction was observable at 1 h and the statistically significant maximal IOP hypotension (–3.4 ± 1.1 mm Hg) was obtained at 4 h post-dosing. When the same quantity of AM404 was applied in a propylene glycol vehicle, no significant decrease in IOP was observed (Fig. 2B). Indeed, the dose induced a statistically significant initial increase of IOP (+3.6 ± 1.4 mm Hg).

Fig. 3A shows that AEA tended to decrease IOP in the presence of PMSF, although AEA did not have a significant effect on IOP when compared to control vehicle. AM404 (125 μg, 50 μl, 15 min prior to AEA) pretreatment did not potentiate or prolong the ocular effects of topical AEA (62.5 μg, 25 μl). A subcutaneous injection of PMSF (24 mg/kg, 30 min prior to AEA), the non-selective serine protease inhibitor that also inhibits fatty acid amide hydrolase (13), was given to prevent enzymatic inactivation of AEA.

Ocular administration of olvanil at a dose of 62.5 μg did not significantly decrease IOP (a maximal IOP reduction –1.6 ± 0.6 mm Hg occurred at 2 h) compared to the propylene glycol vehicle treatment (Fig. 4A). However, olvanil induced a statistically significant IOP reduction (–3.2 ± 1.1 mm Hg at 3 h) at a dose of 312.5 μg, when compared to the control (Fig. 4A).

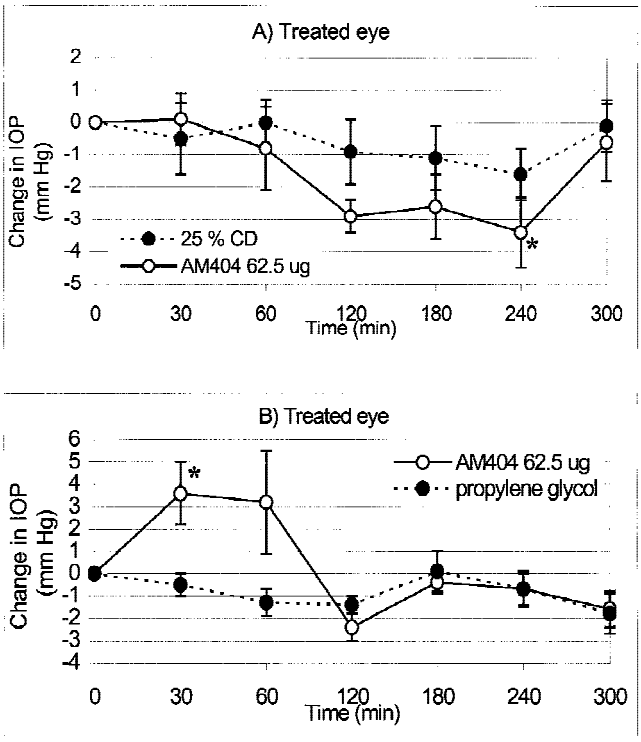

Fig. 2. IOP-changes (mean ± S.E., n = 6) in treated eyes of normotensive rabbits after unilateral ocular administration (25 μl) of AM404 (62.5 μg) in an aqueous 25% HP-β-CD vehicle (Fig. 2A) and in propylene glycol vehicle (Fig. 2B). Asterisks (*) indicate data significantly different from control values (25% HP-β-CD, propylene glycol) at the 95% confidence level (2-tailed paired Student's *t*-test).

Untreated Eye

Unilateral ocular administration of AM404 (62.5 μg) in the aqueous HP-β-CD vehicle, or in propylene glycol, did not cause statistically significant effects on IOP in the contralateral untreated eye, compared to the control vehicle (data not shown). Neither dose of olvanil (62.5 μg or 312.5 μg) significantly affected IOP in the untreated eye (Fig. 4B). As with the treated eyes, AM404 (125 μg) did not affect IOP responses of AEA (62.5 μg) in the untreated eyes (Fig. 3B).

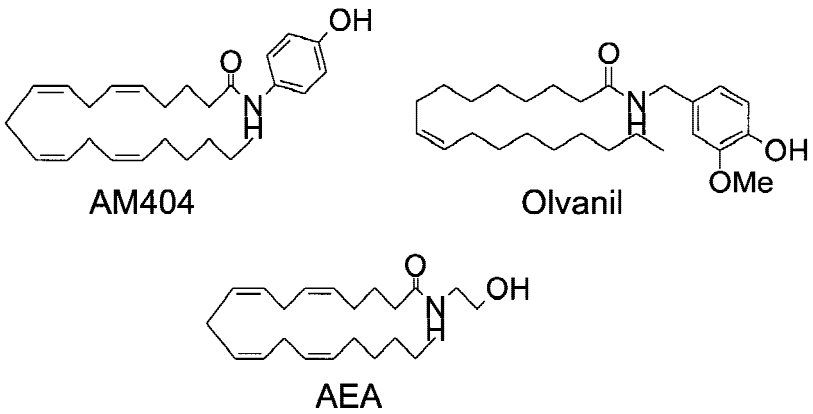

Fig. 1. Chemical structures of anandamide (AEA) and the transport uptake inhibitors, AM404 and olvanil.

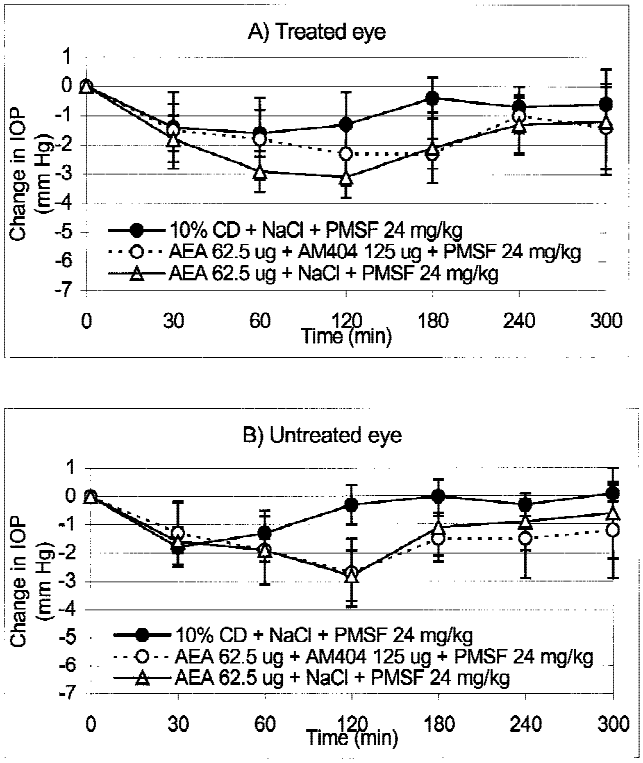

Fig. 3. IOP changes (mean ± S.E., n = 6) in treated (A) and untreated (B) eyes of normotensive rabbits after unilateral ocular administration of AEA (62.5 μg, 25 μl) with (○) and without (Δ) pretreatment with AM404 (125 μg, 50 μl, 15 min prior to AEA) in 25% HP-β-CD vehicle. PMSF (24 mg/kg, s.c.) was given 30 min prior to the AEA. Control treatment: Unilateral ocular application of a 0.9% NaCl solution (50 μl) 15 minutes prior to a 10% HP-β-CD ophthalmic vehicle and subcutaneous PMSF administration (24 mg/kg) 30 min prior to the vehicle (●).

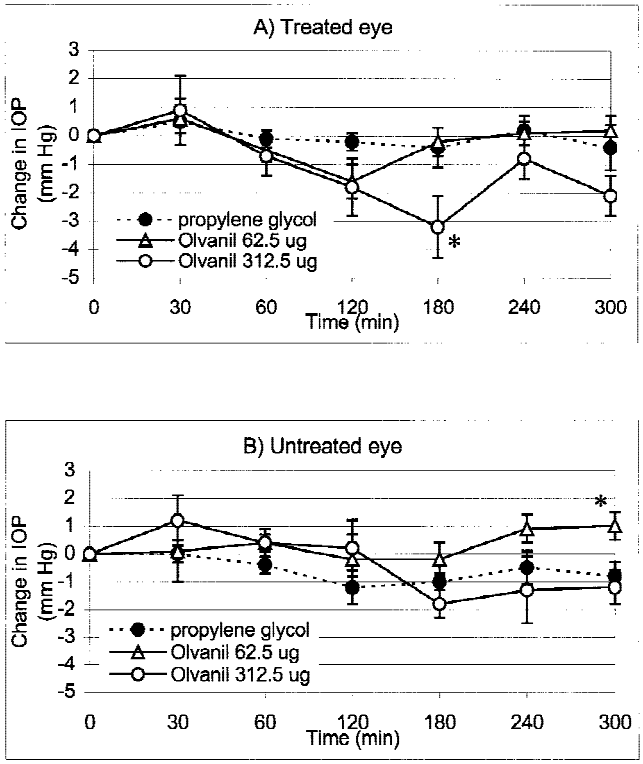

Fig. 4. IOP-changes (mean ± S.E.) in treated (A) and untreated (B) eyes of normotensive rabbits after unilateral ocular administration (25 μl) of 62.5 μg olvanil (Δ) (n = 6), 312.5 μg olvanil (○) (n = 5) or propylene glycol vehicle (●) (n = 5). Asterisks (*) indicate data significantly different from values for vehicle at the 95% confidence level (ANOVA, Fisher's PLSD test).

Autoradiography Study

To assess if AM404 or olvanil exhibit cannabinoid agonist properties, CB1 receptor-dependent G protein activity was monitored using [^{35}S]GTPγS autoradiography of rat brain sections. As evident from Fig. 5, neither compound at concentrations up to 10^{-4} M exhibited agonist activity at the CB1 receptor under these conditions, whereas the nonclassical cannabinoid CP-55,940 (10^{-5} M), which binds with high affinity to the CB1 and CB2 receptors (21), generated a robust response in the various CB1 receptor-enriched regions. The stable AEA analog, *R*-1-methanandamide served as an additional control and, at a 10^{-4} M concentration, generated a weak signal, in the CP-55,940-responsive brain regions, consistent with a partial agonist action (22) at the CB1 receptor. Similar results were obtained using either 0.1% or 2% BSA as a carrier for the compounds, and regardless of whether PMSF (10^{-4} M) was included or not (data not shown). All responses to CP-55,940 and *R*-1-methanandamide were eliminated (data not shown) in the presence of the CB1-selective antagonist SR141716A (10^{-6} M).

DISCUSSION

Beltramo et al. (15) and Calignano et al. (17) have shown that AM404 may potentiate the pharmacological effects of AEA. In contrast, our results indicate that AM404 had a pharmacological effect when administered in the absence of AEA. Further, it did not potentiate or prolong the ocular hypotensive effects of AEA. Consequently, data generated by co-administration of AM404 and AEA may probably be the sum of the combined IOP effects of both compounds. The effect of AM404 pretreatment on the IOP profile induced by AEA was studied only at the AM404 dose of 125 μg due to the fact that the latter compound itself affected IOP (Fig. 3B).

In the present study, subcutaneous pretreatment with PMSF was used because AEA (62.5 μg) evokes a bi-phasic effect (i.e., initial ocular hypertension followed by a significant IOP reduction) in the absence of this FAAH inhibitor (5), probably due to AEA hydrolysis. The resulting arachidonic acid exhibits the same IOP profile, which is eliminated in the presence of an indomethacin pretreatment (6), suggesting prostanoid involvement. As PMSF prevents AEA hydrolysis, only the hypotensive phase is observed (Fig. 2A). Rakhshan et al. (23) recently reported that FAAH inhibitors may decrease AEA transport in RBL-2H3 cells and Maccarone et al. (24) have hypothesized that PMSF may affect the active site of the transporter protein (i.e., PMSF may have similar effects as AEA uptake inhibitors). Consequently, it is reasonable to assume that the synergetic effects of AM404 upon the action of AEA would be enhanced by the absence of PMSF.

It was observed that ophthalmic AM404 and olvanil decrease IOP in the treated eyes of normotensive rabbits. This

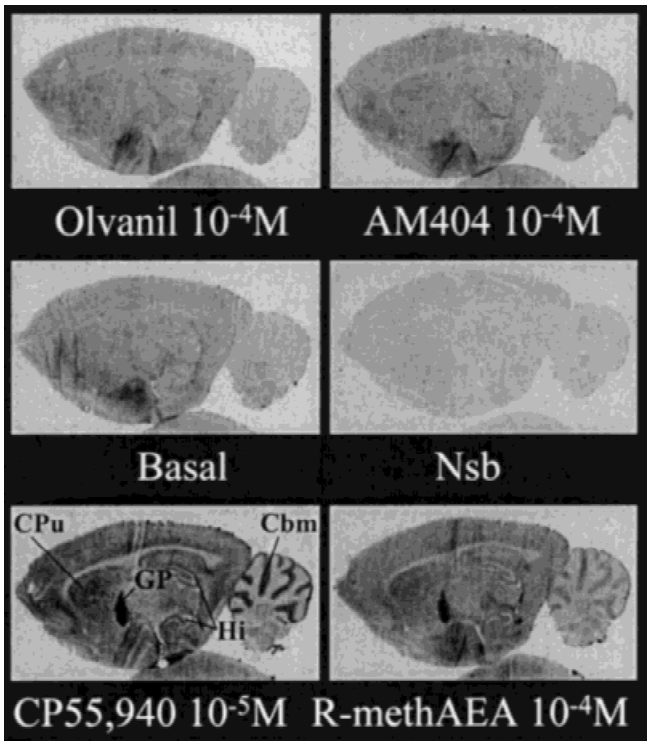

Fig. 5. CB1 receptor-stimulated G proteins were visualized in rat brain sections using [^{35}S]GTPγS autoradiography, as detailed in materials and methods. Note that the synthetic cannabinoid agonist CP-55,940 (10^{-5} M) generates robust responses in various CB1-enriched brain regions (36) such as the globus pallidus (GP) and the molecular layer of the cerebellum (Cbm). A less intense signal is evident in the caudate putamen (CPu), the hippocampus (Hi) and cerebral cortex. *R*-1-methanandamide (*R*-methAEA, 10^{-4} M), a catabolism-resistant analog of AEA, generates weak but detectable responses in CP-55,940-responsive brain regions, thus having the characteristics of a partial agonist (21), whereas AM404 and olvanil (each at 10^{-4} M) exhibit no such agonist activity. Nsb = nonspecific binding.

may be due to prevention of carrier-mediated cellular uptake and subsequent intracellular metabolism of an endogenous AEA acting as a ligand for IOP reduction (5,25). When carrier-mediated cellular uptake of AEA is inhibited, its endogenous levels would be increased in the extracellular space, where the binding sites of CB1 receptors are located (26). Initial results suggest that topical AEA reduces IOP via the CB1 receptor when AEA hydrolysis is prevented by PMSF (unpublished data). In addition, previous study by Pate et al. (8) has indicated this mechanism for other cannabinoids, including an FAAH-resistant AEA analog.

It has been reported that the AEA transporter participates in the cellular uptake of 2-arachidonyl glycerol (2-AG) (27). 2-AG is an endogenous cannabinoid receptor agonist (4), which has been reported to decrease IOP in normotensive rabbits (7) with potency comparable to AEA. Thus, AM404 and olvanil may increase 2-AG levels as well as AEA levels in the eye and reduce IOP via this mechanism. Indeed, 2-AG has been found in rat retina (9), and brain concentrations (28) are approximately 700-fold higher than those of AEA.

Fig. 3 suggests the possibility that prostaglandin synthesis may play a role in mediating the IOP effects of AM404. Although an IOP reduction was observable after administration of 62.5 μg of AM404 in the HP-β-CD vehicle, an initial increase of IOP was observed with the same dose applied via a propylene glycol vehicle. Substantial complexation of a drug with cyclodextrins may diminish ocular absorption of the drug (29), because only the free drug, not the drug-CD complex, can penetrate across the cornea (30), Consequently, it can be assumed that a larger fraction of the administered AM404 was absorbed into the eye from the propylene vehicle. Thus, the present result suggests that high ocular doses of AM404 induced an initial increase of IOP in treated eyes. We have demonstrated earlier that the initial increase of IOP in treated eyes after AEA administration is dose-dependent; the higher the dose, the higher the initial increase of IOP (5). We have shown that an initial increase of IOP after AEA administration is most likely due to enzymatic hydrolysis of AEA and subsequent formation of ocular arachidonic acid (6), a prostanoid metabolic precursor. The present results strongly suggest that AM404 is also degraded to arachidonic acid in the eye, although AM404 is reported to be a poor FAAH substrate (31). Piomelli et al. (27) have observed that AM404 may serve as a substrate for the AEA transporter. Thus, inactivation of AM404 may occur by carrier-mediated transport into cells, followed by enzymatic hydrolysis by FAAH. However, the IOP lowering effects of olvanil are not mediated via a prostanoid mechanism, as olvanil is not an arachidonic acid derivative.

The affinities of AM404 (K_i = 1.8 μM) and olvanil (K_i = 1.6 – 7.1 μM) for the CB1 receptor (32,33) are on same order of magnitude as their respective affinities (AM404 K_i = 2.1 μM, olvanil K_i = 14.1 μM) for the transport protein (16). Consequently, it could be argued that the IOP decreasing effects of AM404 and olvanil are due to their agonist activity at the CB1 receptor. However, our results do not support this, as neither compound at concentrations up to 10^{-4} M exhibited agonist properties at the CB1 receptors. Thus, we conclude that the IOP effects of AM404 or olvanil were likely not mediated through direct agonism at the CB1 receptors. Beltramo et al. (15) have also reported that AM404 does not act in this fashion.

It has been reported recently that AM404 and AEA activate vanilloid receptors (VR1) (34,35). Consequently, it can be argued that the IOP effects of olvanil, AM404 and AEA might be mediated via these receptors. However, it is not currently known whether VR1 receptors are present in the eye or whether they are involved in regulating of IOP.

In conclusion, the present study shows that topical doses of AM404 or olvanil decrease IOP in normotensive rabbits. These results suggest that AEA transport inhibitors may be useful as anti-hypertensive agents in the treatment of glaucoma.

ACKNOWLEDGMENTS

This work was supported by grants from the Academy of Finland, the National Technology Agency (Finland), and the Finnish Cultural Foundation of Northern Savo. The authors thank Prof. Arto Urtti for valuable discussions. Pfizer and Sanofi are gratefully acknowledged for their kind contributions of the CP-55,940 and SR 141716A, respectively.

REFERENCES

1. B. K. Colasanti. Ocular hypotensive effect of marihuana cannabinoids: Correlate of central action or separate phenomenon? *J. Ocul. Pharmacol.* **2**:249–304 (1986).

2. R. S. Hepler and I. M. Frank. Marijuana smoking and intraocular pressure. *J. Am. Med. Assoc.* **217**:1392 (1971).
3. W. A. Devane, L. Hanuš, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, and R. Mechoulam. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**:1946–1949 (1992).
4. R. Mechoulam, S. Ben-Shabat, L. Hanuš, M. Ligumsky, N. E. Kaminski, A. R. Schatz, A. Gopher, S. Almog, B. R. Martin, D. R. Compton, R. G. Pertwee, G. Griffin, M. Bayewitch, J. Barg, and Z. Vogel. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* **50**:83–90 (1995).
5. D. W. Pate, K. Järvinen, A. Urtti, P. Jarho, and T. Järvinen. Ophthalmic arachidonylethanolamide decreases intraocular pressure in normotensive rabbits. *Curr. Eye Res.* **14**:791–797 (1995).
6. D. W. Pate, K. Järvinen, A. Urtti, P. Jarho, M. Fich, V. Mahadevan, and T. Järvinen. Effects of topical anandamides on intraocular pressure in normotensive rabbits. *Life Sci.* **58**:1849–1860 (1996).
7. D. W. Pate, K. Järvinen, A. Urtti, P. Jarho, V. Mahadevan, and T. Järvinen. Effects of topical alpha-substituted anandamides on intraocular pressure in normotensive rabbits. *Pharm. Res.* **14**:1738–1743 (1997).
8. D. W. Pate, K. Järvinen, A. Urtti, V. Mahadevan, and T. Järvinen. Effect of the CB1 receptor antagonist, SR 141716A, on cannabinoid-induced ocular hypotension in normotensive rabbits. *Life Sci.* **63**:2181–2188 (1998).
9. A. Porcella, P. Casellas, G. L. Gessa, and L. Pani. Cannabinoid receptor CB1 mRNA is highly impressed in rat ciliary body: Implications for the antiglaucoma properties of marihuana. *Mol. Brain Res.* **58**:240–245 (1998).
10. A. Straiker, N. Stella, D. Piomelli, K. Mackie, H. J. Karten, and G. Maguire. Cannabinoid CB1 receptors and ligands in vertebrate retina: Localization and function of an endogenous signalling system. *Proc. Natl. Acad. Sci. USA* **96**:14565–14570 (1999).
11. A. J. Straiker, G. Maguire, K. Mackie, and J. Lindsey. Localization of cannabinoid CB1 receptors in the human anterior eye and retina. *Invest. Ophthalmol. Vis. Sci.* **40**:2442–2448 (1999).
12. V. Di Marzo, A. Fontana, H. Cadas, S. Schinelli, G. Cimino, J.-C. Schwarzt, and D. Piomelli. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372**: 686–691 (1994).
13. D. G. Deutsch and S. A. Chin. Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem. Pharmacol.* **46**:791–796 (1993).
14. C. J. Hillard, W. S. Edgemond, A. Jarrahian, and W. B. Campbell. Accumulation of N-arachidonoylethanolamide (anandamide) into cerebellar granule cells occurs via facilitated diffusion. *J. Neurochem.* **69**:631–638 (1997).
15. M. Beltramo, N. Stella, A. Calignano, S. Y. Lin, A. Makriyannis, and D. Piomelli. Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science* **277**:1094–1097 (1997).
16. M. Beltramo and D. Piomelli. Anandamide transport inhibition by the vanilloid agonist olvanil. *Eur. J. Pharmacol.* **364**:75–78 (1999).
17. A. Calignano, G. La Rana, M. Beltramo, A. Makriyannis, and D. Piomelli. Potentiation of anandamide hypotension by the transport inhibitor AM404. *Eur. J. Pharmacol.* **337**:R1–R2 (1997).
18. M. Rinaldi-Carmona, F. Barth, M. Héaulme, D. Shire, B. Calandra, C. Congy, S. Martinez, J. Maruani, G. Néliat, D. Caput, P. Ferrara, P. Soubrié, J. C. Breliére, and G. Le Fur. SR 141716A, a potent and selective antagonist on the brain cannabinoid receptor. *FEBS Lett.* **350**:240–244 (1994).
19. J. T. Laitinen. Selective detection of adenosine A_1 receptor-dependent G protein activity in basal and stimulated conditions of rat brain [^{35}S]guanosine 5′-(thio)triphosphate autoradiography. *Neuroscience* **90**:1265–1279 (1999).
20. J. T. Laitinen and M. Jokinen. Guanosine 5′-(–[^{35}S]thio) triphosphate autoradiography allows selective detection of histamine H_3 receptor-dependent activation of G proteins in rat brain tissue sections. *J. Neurochem.* **71**:808–816 (1998).
21. C. C. Felder, K. E. Joyce, E. M. Briley, J. Mansouri, K. Mackie, O. Blond, Y. Lai, A. L. Ma, and R. L. Mitchell. Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors *Mol. Pharmacol.* **48**:443–450 (1995).
22. T. H. Burkey, R. M. Quock, P. Consroe, F. J. Ehlert, Y. Hosohata, W. R. Roeske, and H. I. Yamamura. Relative efficacies of cannabinoid CB1 receptor agonists in the mouse brain. *Eur. J. Pharmacol.* **336**:295–298 (1997).
23. F. Rakhshan, T. A. Day, R. D. Blakeley, and E. L. Barker. Carrier-mediated uptake of the endogenous cannabinoid anandamide in RBL-2H3 cells. *J. Pharmacol. Exp. Ther.* **292**:960–967 (2000).
24. M. Maccarone, M. van der Stelt, A. Rossi, G. A. Veldink, J. F. G. Vliegenthart, and A. F. Agrò. Anandamide hydrolysis by human cells in culture and brain. *J. Biol. Chem.* **273**:32332–32339 (1998).
25. Y. Mikawa, S. Matsuda, T. Kanagawa, T. Tajika, N. Ueda, and Y. Mimura. Ocular activity of topically administered anandamide in the rabbit. *Jpn. J. Ophthalmol.* **41**:217–220 (1997).
26. W. A. Devane, F. A. Dysarz III, M. R. Johnson, L. S. Melvin, and A. C. Howlett. Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.* **34**:605–613 (1988).
27. D. Piomelli, M. Beltramo, S. Glasnapp, S. Y. Lin, A. Goutopoulos, X.-Q. Xie, and A. Makriyannis. Structural determinants for recognition and translocation by the anandamide transporter. *Proc. Natl. Acad. Sci. USA* **96**:5802–5807 (1999).
28. T. Sugiura, S. Kondo, A. Sukagawa, S. Nakane, A. Shinoda, K. Itoh, A. Yamashita, and K. Waku. 2-Arachidonoylgylcerol: A possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun*. **215**:89–97 (1995).
29. P. Jarho, K. Järvinen, A. Urtti, V. J. Stella, and T. Järvinen. The use of cyclodextrins in ophthalmic formulations of dipivefrin. *Int. J. Pharm.* **153**:225–233 (1997).
30. H. W. Frijlink, A. C. Eissens, A. J. M. Schoonen, and C. F. Lerk. The effect of cyclodextrins on drug absorption. II. *In vivo* observation. *Int. J. Pharm.* **64**:195–205 (1990).
31. W. Lang, C. Qin, S. Lin, A. D. Khanolkar, A. Goutopoulos, P. Fan, K. Aboudzid, Z. Meng, D. Biegel, and A. Makriyannis. Substrate specificity and stereoselectivity of rat brain microsomal anandamide amidohydrolase. *J. Med. Chem.* **42**:896–902 (1999).
32. A. D. Khanolkar, V. Abadji, S. Lin, A. G. Hill, G. Taha, K. Abouzid, Z. Meng, P. Fan, and A. Makriyannis. Head group analogs of arachidonyl-ethanolamide, the endogenous cannabinoid ligand. *J. Med. Chem* **39**:4515–4519 (1996).
33. V. Di Marzo, T. Bisogno, D. Melck, R. Ross, H. Brockie, L. Stevenson, R. Pertwee, and L. De Petrocellis. Interactions between synthetic vanilloids and the endogenous cannabinoid system, *FEBS Lett.* **436**:449–454 (1998).
34. P. M. Zygmunt, H. Chuang, P. Movahed, D. Julius, and E. D. Högestätt. The anandamide transport inhibitor AM404 activates vanilloid receptors, *Eur. J. Pharmacol.* **396**:39–42 (2000).
35. D. Smart and J. C. Jerman. Anandamide: an endogenous activator of the vanilloid receptor. *Trends Pharmacol. Sci.* **21**:134 (2000).
36. M. Herkenham, A. B. Lynn, M. R. Johnson, L. S. Melvin, B. R. de Costa, and K. C. Rice. Characterization and localization of cannabinoid receptors in rat brain: A quantitative *in vitro* autoradiographic study. *J. Neurosci.* **11**:563–583 (1991).