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Cannabinoid CB_1 receptor expression, activation and detection of endogenous ligand in trabecular meshwork and ciliary process tissues

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Abstract

Elevated intraocular pressure is the primary risk factor for glaucoma. Cannabinoids interact with molecular targets in the eye and lower intraocular pressure by an unknown mechanism. The purpose of the present study was to examine eye tissues for functional cannabinoid receptors of the neuronal, CB_1 class, and an endogenous ligand, anandamide. The trabecular meshwork and ciliary processes are the primary structures of the eye that contribute to intraocular pressure and thus were our focus. Total RNA, frozen sections, cellular membranes and primary cultures of cells were prepared from both bovine and cadaveric human tissues. Using cannabinoid CB_1 receptor-specific oligodeoxynucleotide primers, cannabinoid CB_1 receptor antiserum, and cannabinoid-specific compounds (CP-55,940, WIN55,212-2 and SR-141716A), the presence of cannabinoid CB_1 receptors in ciliary processes and trabecular meshwork was determined. Using reverse transcription-polymerase chain reaction, we identified mRNA encoding cannabinoid CB_1 receptor protein in ciliary process and trabecular meshwork cells. Specific binding of anti-CB_1 immunoglobulin-G in tissue sections localized cannabinoid CB_1 receptor protein to the non-pigmented epithelial cells of the ciliary process and cells of the trabecular meshwork. While CP-55,940 and WIN55,212-2 failed to stimulate [^{35}S]GTPγS binding in membrane preparations from trabecular meshwork and ciliary process, CP-55,940 significantly stimulated whole cell [^{35}S]GTPγS binding by 51% over basal in ciliary process epithelial cells and 69% over basal in trabecular meshwork cells permeabilized with 5 μM digitonin ($p < 0.001$). Specificity of agonist stimulation was verified by complete blockade with the specific cannabinoid CB_1 receptor antagonist, SR-141716A. Moreover, activation of cannabinoid CB_1 receptors by CP-55,940 resulted in a 2.3 ± 0.3 and 1.7 ± 0.3-fold stimulation of cAMP accumulation in trabecular meshwork and ciliary process cells, respectively ($p < 0.01$). Lastly, anandamide was detected in human trabecular meshwork (3.08 pmol/g), ciliary process (49.42 pmol/g) and neurosensory retinal (4.48 pmol/g) tissues. These data, for the first time, demonstrate in a single study the presence of both CB_1 mRNA and protein in trabecular meshwork and ciliary processes from two different species. Activation of heterotrimeric G-proteins and stimulation of cAMP accumulation by cannabinoids in vitro suggest that their intraocular pressure-lowering effects in vivo result from activation of cannabinoid CB_1 receptors in the trabecular meshwork and increase aqueous outflow.

Keywords: Anandamide; cAMP; G-protein; Glaucoma; GTPγS; Intraocular pressure

1. Introduction

Elevated intraocular pressure is the primary risk factor for glaucoma, a blinding group of diseases that affects at least 70 million people worldwide (Leske, 1983; Quigley, 1996; Schappert, 1995). Currently, intraocular pressure in those with glaucoma is managed therapeutically with pharmaceuticals that target receptors on the ciliary process or in the outflow pathway, inhibiting aqueous humor secretion or increasing outflow facility, respectively. Interest in cannabinoids as anti-glaucoma agents began 30 years ago with a report documenting intraocular pressure-lowering effects of marijuana in humans (Hepler and Frank, 1971).

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Since this original observation, several studies have demonstrated intraocular pressure-lowering effects of cannabinoids, endogenous, exogenous and synthetic, in several mammalian models regardless of route of administration (Beilin et al., 2000; Colasanti et al., 1984a,b,c; Mikawa et al., 1997; Naveh et al., 2000; Pate et al., 1995, 1996, 1997; Song and Slowey, 2000). The CB_1 class of cannabinoid receptor was implicated because cannabinoids effect on intraocular pressure was blocked with the specific CB_1 receptor antagonist, SR141716A (Pate et al., 1998; Song and Slowey, 2000) however, the cannabinoid site of action is not known.

Both messenger RNA and protein for cannabinoid receptors have been found in many tissues of the eye. Messenger RNA encoding the cannabinoid CB_2 class of cannabinoid receptors was limited to the neurosensory retina of rat by in situ hybridization and reverse transcription-polymerase chain reaction (RT-PCR) (Lu et al., 2000). In addition to the neurosensory retina, messenger RNA encoding the cannabinoid CB_1 class of receptors have been found in the choroid, iris and ciliary body of rat (Porcella et al., 1998). Cannabinoid CB_1 receptor protein was detected in many tissues of the human eye using anti-CB_1 receptor IgG (Straiker et al., 1999a,b). Antibody binding was detected in several regions of the neurosensory retina, including synapses of inner and outer plexiform layers, as well as the inner nuclear and ganglion layers. Moreover, antibodies bound to ciliary epithelium, ciliary muscle, blood vessels of ciliary body, sphincter papillae, corneal epithelium, trabecular meshwork and Schlemm's canal. Thus, the two primary structures of the eye which contribute to intraocular pressure, the ciliary process (aqueous humor formation) and the trabecular meshwork (aqueous humor removal), appear to express cannabinoid CB_1 receptors.

The purpose of the present study was to examine tissues from these two principle structures responsible for the maintenance of intraocular pressure for functional cannabinoid CB_1 receptors and for an endogenous ligand, anandamide. For the first time in a single study, we present data showing the presence of both messenger RNA and protein for cannabinoid CB_1 receptors in cells isolated from ciliary process and trabecular meshwork tissues of two different species. Moreover, we demonstrate that cannabinoid ligands bind receptors, activate heterotrimeric guanine nucleotide-binding proteins (G-proteins) and stimulate the cAMP second messenger pathway in these cells. Lastly, we show that anandamide is present in both trabecular meshwork and ciliary process tissues.

2. Materials and methods

2.1. Human and bovine eyes

Human cadaveric eyes (non-glaucomatous) were obtained from Donor Network of Arizona, the San Diego Eye Bank or Lions Eye Bank of Central Florida < 48 h postmortem. Bovine eyes were obtained from a local slaughterhouse 1–2 h postmortem.

2.2. Human and bovine ciliary epithelial tissue and cells

Human and bovine eyes were cleaned of connective tissue and muscle, and the eye was bisected along its equator. The anterior half was placed cornea-side down in a dissecting dish, and the lens and capsule were removed. Tips were dissected from ciliary processes as described before (Anthony et al., 2000) and snap-frozen for RT-PCR studies and anandamide measurements, embedded in OCT compound (Tissue Tek) for immunofluorescence studies or placed in Hank's Balanced Salt Solution containing penicillin/streptomycin (100 μg/ml) in preparation for cell isolation. Bovine ciliary epithelial cells were isolated from ciliary tips using a trypsin digestion method as described previously (Anthony et al., 2000). Cells were maintained in Dulbecco's Modified Eagle Medium (Life Technologies) containing 10% fetal bovine serum (Life Technologies), 170 mM glutamine and penicillin/streptomycin (100 μg/ml, Life Technologies) in humidified air containing 5% carbon dioxide at 37 °C.

2.3. Human and bovine trabecular meshwork tissue and cells

Trabecular meshwork tissue was isolated from non-glaucomatous human cadaveric eyes using a blunt dissection technique as previously described (Stamer et al., 1995b). Strips of trabecular meshwork tissue were either snap-frozen in liquid nitrogen for RNA isolation and anandamide measurements, embedded in OCT compound and frozen in a dry-ice/ethanol bath for immunofluorescence studies or digested with collagenase for trabecular meshwork cell isolations as detailed previously (Stamer et al., 1995b).

2.4. Total RNA isolation

Total RNA was isolated from confluent cultures of trabecular meshwork or ciliary process cells in 10 cm culture plates using Trizol reagent according to the manufacturer's recommendations (Life Technologies). Following extraction, RNA was precipitated using ice-cold ethanol, resuspended in diethyl pyrocarbonate (Sigma)-treated water and incubated with RNAse-free DNAse (Promega) to digest genomic DNA. RNA was extracted from DNAse using phenol/chloroform, precipitated with ethanol and resuspended in diethyl pyrocarbonate-treated water. Integrity of RNA was verified following separation by electrophoresis into an 0.8% agarose gel containing formaldehyde and visualization using ethidium bromide.

2.5. Reverse transcription-polymerase chain reaction studies

The presence of messenger RNA encoding the CB_1 class of cannabinoid receptor in ciliary process and trabecular meshwork cells was determined by RT-PCR. Utilizing Avian Myeloblastosis Virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN), DNA copies of total RNA were made using oligo dT primers or a primer specific for CB_1 antisense RNA transcripts (5′-CACAGAGCCTCGGCAGA-3′), respectively, as described previously (Stamer et al., 1995a). Amplification of RT cDNA by taq polymerase (Life Technologies) was performed as previously described (Stamer et al., 1995a) using 30–40 PCR cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s). Specific amplification of CB_1 cDNAs was accomplished using a primer set that corresponds uniquely to the cannabinoid CB_1 receptor (sense: 5′-ATGGCCTTGCAGATACCA-3′; antisense: 5′-CACAGAGCCTCGGCAGA-3′) and PCR products were analyzed on 1.4% agarose gels. Based upon published sequence, the expected size of a CB_1 PCR product is 1416 nucleotides. A plasmid containing DNA that encodes the human cannabinoid CB_1 receptor, pCDNA/CB_1 was used as a positive control (source: H.I. Yamamura). Since DNA coding CB_1 contains no introns, we were rigorous with controls to minimize the possibility of false-positive amplification of genomic DNA. Negative controls include: the exclusion of cDNA from PCR (water control), treatment of RNA with RNAse-A prior to RT reaction and the inclusion of RNA not reversed transcribed in the PCR reaction.

2.6. Immunofluorescence microscopy studies

Indirect immunofluorescence microscopy was performed on fresh frozen ciliary process and trabecular meshwork tissues. Tissue samples were embedded in OCT compound, frozen in a dry-ice/ethanol bath and sectioned (8 μm). Sections on slides were fixed in 4% paraformaldehyde in phosphate-buffered saline and incubated overnight with polyclonal antiserum (1:50 dilution, Cayman Chemical) that was raised in rabbits against a synthetic peptide corresponding to the first 14 amino acids of the mammalian cannabinoid CB_1 receptor (MKSILDGLADTTFR). Goat serum (10%, Sigma) and triton X-100 (0.1%, Sigma) were included in incubations to inhibit non-specific binding of antibodies to tissues. Following antibody incubations, tissue sections were washed extensively (4×10 ml $\times$ 15 min) in phosphate-buffered saline containing 0.1% triton X-100. Specific binding of antibodies to receptor was detected using fluorescein (FITC)-conjugated goat anti-rabbit immunoglobulin-G (IgG) at a 1:1000 dilution (Jackson Immunoresearch Laboratories). Tissue sections were incubated with secondary antibodies for 2 h and washed extensively before viewing. Labeled tissue sections were visualized and photographed using an Olympus IX70 inverted fluorescence microscope. Viability of tissue samples in all cases was verified using affinity-purified anti-aquaporin-1 IgG (Stamer et al., 1996b) (1:5000 dilution). Aquaporin-1 was used as a control because it is an integral membrane protein expressed in both tissues studied. Antiserum dilutions for CB_1 antiserum were determined empirically using Chinese hamster ovary (CHO) cells stably expressing human cannabinoid CB_1 receptor and untransfected CHO cells were used as control (Landsman et al., 1997).

2.7. [^{35}S]GTPγS binding studies

Cannabinoid stimulation of [^{35}S]GTPγS binding to membrane homogenates were performed essentially as described before (Hosohata et al., 1997). Eye and brain tissues were homogenized in 20 volumes of ice-cold TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4). Homogenates were centrifuged at $40{,}000 \times g$ at 4 °C for 15 min, and the pellet was resuspended in 20 volumes of assay buffer (25 mM Tris–HCl, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 50 μM GDP, 30 μM bestatin, 10 μM captopril and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). Following a preincubation at 30 °C for 30 min, membranes were centrifuged then resuspended in fresh assay buffer to an optical density$_{280}$ (OD_{280}) of 0.1 (human ciliary process and trabecular meshwork and bovine trabecular meshwork) or 0.8 (bovine ciliary process and mouse brain). Membranes were incubated with 1 μM of WIN55,212-2 or CP-55,940 in the presence of 0.1 nM [^{35}S]GTPγS (1250 Ci/mmol, New England Nuclear, Boston, MA) and 0.25% of bovine serum albumin in a total volume of 1.0 ml. After 90 min incubation at 30 °C, the reaction was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by four washes with 4 ml ice-cold 25 mM Tris/120 mM NaCl, pH 7.4. Bound radioactivity was measured by liquid scintillation spectrophotometry after an overnight extraction with EcoLite® scintillation cocktail.

Binding of [^{35}S]GTPγS (1250 Ci/mmole) in permeabilized trabecular meshwork and ciliary process cells was measured using a modification of previously published methods (Varga et al., 2000). Cells were seeded onto 24-well culture plates and maintained in humidified air containing 5% CO_2 at 37 °C. Cells were washed twice with Iscove's modified Dulbecco's medium and incubated with 1 ml permeabilization buffer (25 mM Tris–HCl, 150 mM NaCl, 2.5 mM $MgCl_2$, 1.0 mM EDTA, 5 μM digitonin) for 15 min at 37 °C. Permeabilization buffer was replaced with assay buffer (25 mM Tris–HCl, 150 mM NaCl, 2.5 mM $MgCl_2$, 1.0 mM EDTA, 50 μM GDP, 5 μM digitonin) containing [^{35}S]GTPγS alone (0.5 nM), [^{35}S]GTPγS (0.5 nM) plus CP-55,940 (1–10 μM) or [^{35}S]GTPγS (0.5 nM), CP-55,940 (10 μM) plus the CB-1 antagonist SR141716A (1 μM) and incubated for 30 min at 37 °C. The incubation was terminated upon aspiration of

assay buffer and washing cells with 1 ml ice-cold wash buffer (25 mM Tris–HCl, 120 mM NaCl). Cell membranes were extracted with 0.5 ml 10% SDS (overnight) and transferred into Ecolite liquid scintillation cocktail. Radioactivity was measured in a Beckman LS 6000SE liquid scintillation spectrophotometer. [^{35}S]GTPγS was purchased from Dupont NEN (Boston, MA) and GDP from Boehringer Mannheim. CP-55,940 was generously provided by Pfizer (Groton, CT) and SR141716A from Gerard Le Fur (Sanofi Recherche, Montpellier, France).

2.8. Measurement of anandamide in human cadaveric eye tissues

The procedures used for tissue extraction and quantification of anandamide are based upon a previously described method (Felder et al., 1996). Strips of trabecular meshwork tissue, tips of ciliary process tissue and whole neurosensory retinas were dissected from human cadaveric eyes and snap-frozen in liquid nitrogen. Like tissues were pooled and extracted with organic solvent (chloroform and methanol), and further purified by solid phase extraction. Samples were chromatographically separated on a Eclipse XDB C-18 column (Zorbax) using a methanol/ammonium acetate mobile phase, and the amount of anandamide was quantified using a API 3000 (Sciex) in positive APCI mode. Sample concentrations of anandamide were based on the response of a [$^{2}H_{8}$] anandamide internal standard and a linear standard curve run concurrently with tissue samples. Tissue concentrations of anandamide are reported as picomoles of anandamide per gram of tissue.

2.9. Cyclic AMP studies

Intracellular cAMP was measured using a protein kinase A binding assay as described previously with few modifications (Stamer et al., 1996a). Human trabecular meshwork, ciliary process, NG108-15 or Chinese hamster ovary cells were seeded onto 24-well plates at a density of 50,000 to 100,000 cells per well and were maintained as described above. Cells were washed with serum-free Dulbecco's modified eagle medium and maintained in serum-free Dulbecco's modified eagle medium overnight. After replacement with fresh serum-free medium buffered with *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES), cells were preincubated with 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) for 1 min at 37 °C. In blocking experiments, cells were preincubated for 30 min with the CB_1 antagonist, SR141716A, prior to addition of IBMX or drugs. The medium was aspirated and drugs plus 0.5 mM IBMX were added for 15 min. The drug solutions were aspirated, the plates were transferred to ice, and 150 μl of ice-cold Tris/EDTA buffer (50 mM Tris/4 mM EDTA, pH 7.5) was added to each well. Cells were scraped, transferred to microfuge tubes, boiled for 10 min and centrifuged in a microcentrifuge at maximum speed for 2 min ($\sim 12{,}000 \times g$). A fraction of the supernatants (50 μl) was added to 50 μl [^{3}H]cAMP (0.9 pmol/50 μl, NEN, Boston, MA) and 100 μl of cold PKA solution (0.06 mg PKA/ml of Tris/EDTA, Sigma). Following a 2 h incubation at 4 °C, 100 μl of activated charcoal solution (20 mg/ml activated charcoal in Tris/EDTA containing 2% BSA) was added and the mixture, vortexed and centrifuged at maximum speed in a microcentrifuge for 1 min at 22 °C. Samples were placed on ice and 200 μl of each supernatant was transferred to scintillation vials for counting. A standard curve was generated by adding 50 μl of cAMP standards (0.25–32.0 pmol, Sigma), instead of cytosol, to protein kinase A solution with [^{3}H]cAMP.

2.10. Data analysis

Data were analyzed using Prism 3.0 software (GraphPad Software, San Diego, CA). The differences between basal and CP-55,940-stimulated [^{35}S]GTPγS binding in the absence or presence of SR141716A were statistically determined by one-way ANOVA, followed by Newman–Kewls test. Significant differences between experimental conditions of cAMP studies were determined using a two-tailed Student's *t*-test assuming unequal variance.

3. Results

Four independent methods were used to determine the presence of cannabinoid CB_1 receptors in the structures of the mammalian eye that influence intraocular pressure. Using ciliary process tips and trabecular meshwork strips carefully dissected from human and bovine eyes, RT-PCR was used to determine presence of messenger RNA and the specific binding of anti-CB_1 receptor immunoglobulin-G or synthetic cannabinoid compounds to cannabinoid CB_1 receptors were used to determine the presence of cannabinoid CB_1 receptor protein. Stimulation of [^{35}S]GTPγS binding and cAMP accumulation by synthetic cannabinoid compounds was used to assess functional coupling of cannabinoid CB_1 receptor to heterotrimeric G-proteins in live cells prepared from ciliary process and trabecular meshwork tissues. Lastly, liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) was used to determine anandamide levels in the trabecular meshwork, ciliary process and retina.

3.1. RT-PCR studies

DNA copies of ciliary process and trabecular meshwork mRNAs were made using reverse transcriptase and oligo dT primers or primers specific for CB_1 mRNA. Using oligodeoxynucleotides that correspond specifically to the amino and carboxyl termini of human cannabinoid CB_1 receptor, analysis of cDNAs by PCR revealed the presence of full-length CB_1 mRNA in ciliary process and trabecular

meshwork tissues and primary cells prepared from these tissues. The amplification of DNA of the appropriate size (1416 base pairs) for cannabinoid CB_1 receptor is shown in Fig. 1. While PCR products from ciliary process tissue and cells were consistently reproducible, products from trabecular meshwork tissue were variable, giving either no or weak products that usually required 40 PCR cycles to visualize (panel B, lane 2 versus lane 8). Following additional PCR cycles, no products appeared in the control lanes (not shown). RNA integrity was verified by formaldehyde/agarose gel electrophoresis and cDNA integrity was verified by PCR amplification of full-length aquaporin-1 from all cDNA libraries. The presence of a cannabinoid CB_1 receptor isoform, CB_{1a}, was examined in lane 8 of panel A by loading 4 × of PCR product shown in lane 2. An expected product of 1233 base pairs for CB_{1a} receptor, having a 183 base pair internal deletion from CB_1, was not detected.

3.2. Immunofluorescence microscopy studies

Tips of ciliary process and strips of trabecular meshwork were dissected from cadaveric human and bovine tissues, embedded in OCT compound and sectioned for indirect immunofluorescence studies. The presence of cannabinoid CB_1 receptors in these tissue sections was investigated using antibodies raised in rabbits against the first 14 amino acids of the mammalian cannabinoid CB_1 receptor. Fig. 2 shows specific decoration (green fluorescence) of non-pigmented ciliary epithelial cells with FITC-conjugated secondary antibodies in both human (panel A) and bovine (panel D) ciliary process tissues. Labeling of the integral membrane protein, aquaporin-1, was used in sequential sections to identify the non-pigmented epithelial layer and as a control to verify viability of all tissues (panels B and E in Figs. 2 and 3). Fig. 3 demonstrates the specific labeling (green fluorescence) of trabecular meshwork cells on collagen beam surfaces in both human (panel A) and bovine (panel D) preparations. In order to indicate non-specific binding of FITC-conjugated goat anti-rabbit IgG to tissues as well as endogenous autofluorescence, primary antibodies were omitted (panels C and F in Figs. 2 and 3).

3.3. [^{35}S]GTPγS studies

Membranes were prepared from ciliary process and trabecular meshwork tissue dissected from cadaveric human and bovine eyes and primary cells isolated from tissues. Standard radioligand binding studies were performed and specific binding was *not detected* in any of these preparations examined at three different tissue concentrations using two different radiolabeled cannabinoid compounds, [^{3}H]-CP-55,940 and [^{3}H]-SR141716A in four individual experiments (data not shown). In an attempt to demonstrate functional coupling of CB_1 receptors to G-proteins, membrane homogenates containing equivalent total protein were incubated with CP-55,940 or WIN55,212-2 (1–10 μM) and analyzed for agonist-stimulated binding of [^{35}S]GTPγS. No cannabinoid agonist stimulation of [^{35}S]GTPγS was found in any of these preparations in two

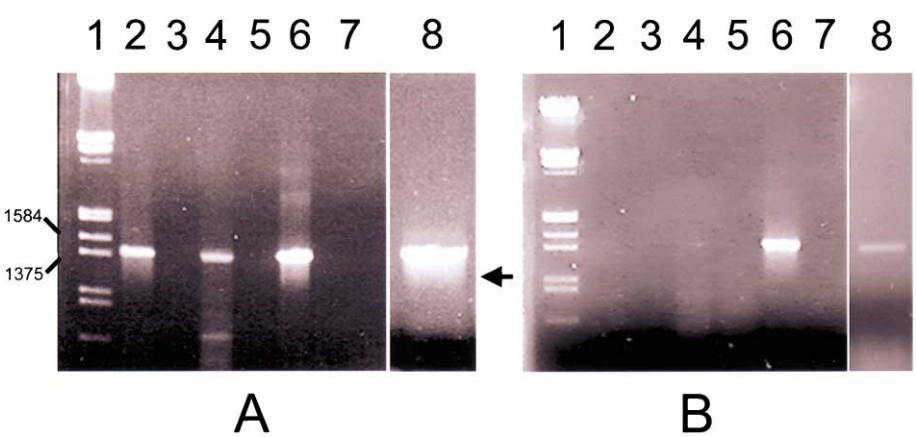

Fig. 1. Photographs of two ethidium-stained agarose gels containing the products of RT-PCR using primers specific for the CB_1 class of cannabinoid receptors. Panel A: Lane 1 contains molecular size standards; lanes 2–5 show products of reactions using mRNA from ciliary process cells (1416 bp). mRNAs used in lanes 2–3 were transcribed using oligo dT deoxynucleotides, while lanes 4–5 used CB_1 receptor-specific antisense oligodeoxynucleotides. As a control for genomic DNA contamination, messenger RNA templates shown in lanes 3 and 5 were treated with RNAse-A prior to RT-PCR. Lane 6 is a positive control utilizing pcDNA/CB_1 as a template. As a negative control, template was omitted in lane 7. The presence of the CB_{1a} receptor subtype was specifically examined in lane 8 by loading 4 × of PCR product shown in lane 2 (arrow indicates the predicted size of a CB_{1a} PCR product, 1233 bp). Panel B: Lane designations in Panel B (lanes 1–7) are identical to that shown in Panel A, except reactions shown in Panel B utilized mRNAs isolated from human trabecular meshwork cells. Using RNA isolated from human trabecular meshwork cells, lane 8 shows a PCR product that required 40 PCR cycles to visualize.

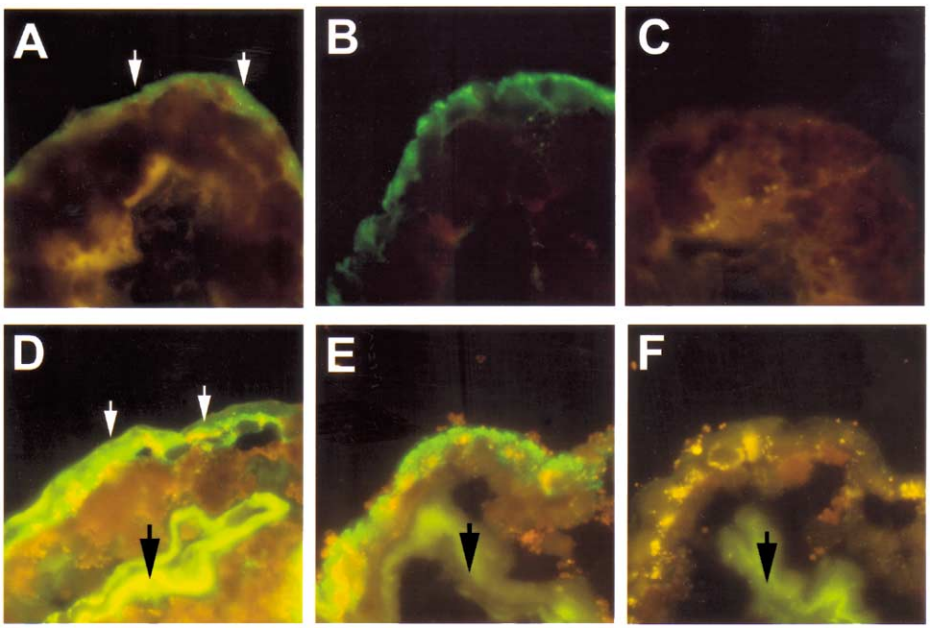

Fig. 2. Immunofluorescence localization of CB_1 receptors in human and bovine ciliary process tissues. Shown are photomicrographs of frozen sections of human (Panels A–C) and bovine (Panels D–F) ciliary process tissues. Panels A and D demonstrate specific reactivity of anti-CB_1 receptor antiserum to cells of the ciliary process (green fluorescence, white arrows). Labeling of aquaporin-1 (Panels B and E) was included as positive control to demonstrate tissue viability. Panels C and F show non-specific labeling (yellow autofluorescence, black arrows) in tissues untreated with primary antibodies.

independent experiments (Fig. 4). Membrane homogenates from rat brains were used as a positive control for these assays and agonist stimulation resulted in 90% of basal [^{35}S]GTPγS binding.

In additional studies, primary cultures of cells from ciliary process and trabecular meshwork tissues were permeabilized with digitonin, stimulated with cannabinoids and examined for ^{35}S-GTPγS binding. Fig. 5 demonstrates that [^{35}S]GTPγS binding was significantly stimulated by CP-55,940 in primary cultures of permeabilized cells from ciliary process and trabecular meshwork tissues ($p < 0.001$). [^{35}S]GTPγS binding increased 51% ± 14 over control in ciliary process cells (panel A) and 69% ± 15 in trabecular meshwork cells (panel B). In both cases, CP-

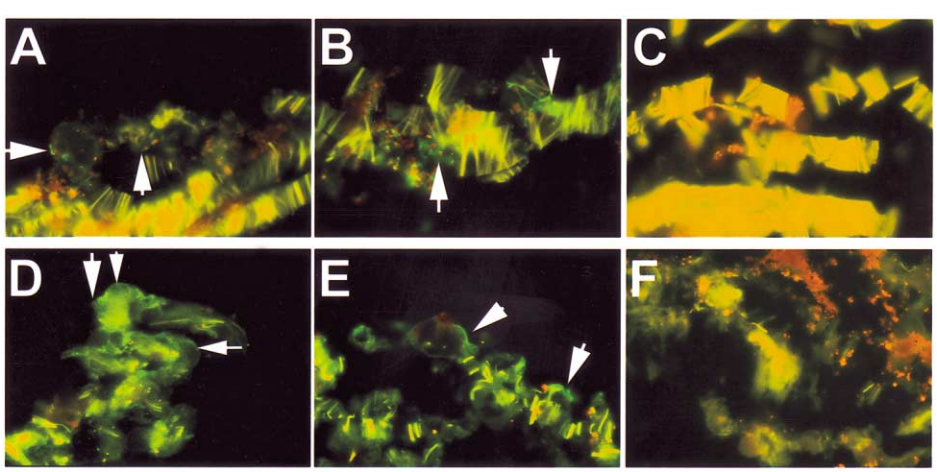

Fig. 3. Immunofluorescence localization of CB_1 receptors in human and bovine trabecular meshwork tissues. Shown are photomicrographs of frozen sections of human (Panels A–C) and bovine (Panels D–F) trabecular meshwork tissues. Panels A and D show specific reactivity of anti-CB_1 receptor antiserum to cells of the trabecular meshwork (green fluorescence, white arrows). Anti-aquaporin-1 IgG was used in experiments (Panels B and E) as positive controls to demonstrate tissue viability. Panels C and F demonstrate background labeling (yellow autofluorescence) in the absence of primary antibodies.

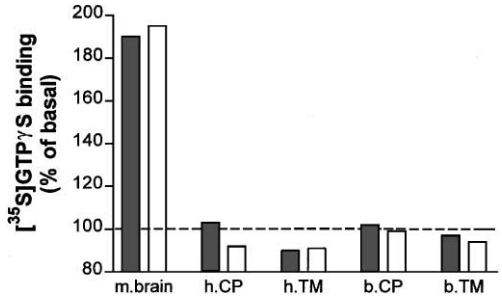

Fig. 4. CB_1 receptor agonist (WIN55,212-2 or CP-55,940)-stimulated [^{35}S]GTPγS binding in membranes prepared from human or bovine eye tissues. Eye tissue were normalized to total protein and incubated with 1 μM WIN55,212-2 (shaded bars) or 1 μM CP-55,940 (white bars) in the presence of 0.1 nM [^{35}S]GTPγS. No stimulation above baseline was observed for any of the eye tissues (hCP = human ciliary process, hTM = human trabecular meshwork, bCP = bovine ciliary process and bTM = bovine trabecular meshwork). The average value of basal [^{35}S]GTPγS binding in the absence of agonist was 582 CPM (hCP), 647 CPM (hTM), 18,383 CPM (bCP) and 546 CPM (bTM). Membranes prepared from mouse brain served as a positive control for the assay.

55,940-stimulated [^{35}S]GTPγS binding was blocked completely with a specific CB_1 antagonist, SR141716A. Alone, SR141716A was without significant effect ($p > 0.001$).

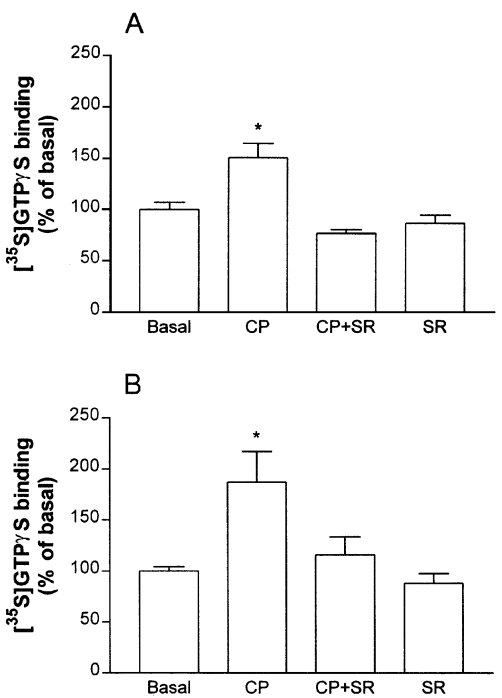

Fig. 5. CB_1 receptor agonist (CP-55,940)-stimulated [^{35}S]GTPγS binding in permeabilized CP and TM cells. Cells in 24-well culture plates were washed and permeabilized with buffer containing 5 μM digitonin. Cells were incubated with 0.5 nM [^{35}S]GTPγS and 10 μM CP-55,940 (CP) in the presence or absence of 1 μM SR141716A (SR) for 30 min. Controls include incubation of cells with 0.5 nM [^{35}S]GTPγS alone or with 1 μM SR141716A. Panel A shows the results obtained using primary cultures of cells isolated from bovine ciliary processes. The average value of basal [^{35}S]GTPγS binding in the absence of agonist was 1088 CPM. Panel B shows the results obtained using primary cultures of cells isolated from a human trabecular meshwork. The average value of basal [^{35}S]GTPγS binding in the absence of agonist was 1209 CPM. Shown for each cell type is one representative experiment of four total performed in triplicate.

3.4. cAMP studies

To explore the possible association of cannabinoid CB_1 receptors with adenylyl cyclase, the effect of CP-55,940 and WIN55212-2 on cAMP accumulation in human trabecular meshwork and ciliary process cells was examined. Chinese hamster ovary (CHO) cells stably expressing recombinant cannabinoid CB_1 receptor and NG10815 cells

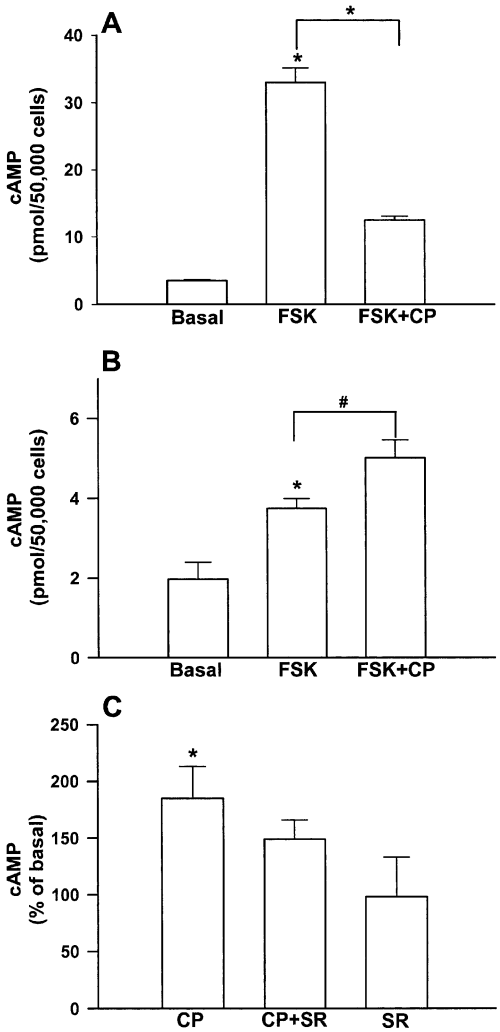

Fig. 6. The effect of the cannabinoid receptor agonist, CP-55,940, on cAMP accumulation in Chinese hamster ovary and human trabecular meshwork cells. The top two panels demonstrate the differential effects of CP-55,940 (CP, 1 μM) on forskolin (FSK)-stimulated cAMP accumulation in Chinese hamster ovary cells stably expressing recombinant cannabinoid CB_1 receptor (Panel A, FSK = 1 μM) and human trabecular meshwork cells (Panel B, FSK = 10 μM). Panel C shows the effect of CP-55,940 (1 μM) alone, without forskolin, or when co-incubated with the CB_1-specific receptor antagonist, SR141716A (1 μM). Data in Panel C are combined from four experiments (±standard error of the mean) and are presented as the percentage of basal levels of cAMP for each condition. Significant differences were found ($^{\#}p < 0.05$, $^{*}p < 0.01$) using a Student's *t*-test assuming unequal variance. Panel A shows one representative experiment of two total performed in sextuplet (±standard error of the mean). Panel B shows one representative experiment of six total performed in quintuplet (±standard error of the mean).

Table 1
Anandamide levels in human cadaveric eye tissues

	Neurosensory retina	Ciliary process	Trabecular meshwork
pmol/g tissue	4.48	49.42	3.08

that express endogenous cannabinoid CB_1 receptor were utilized as controls for these studies. Panel A of Fig. 6 shows the effect of CP-55,940 (1 μM) on forskolin-stimulated (1 μM) cAMP accumulation in CHO cells stably expressing recombinant cannabinoid CB_1 receptor. As expected, CP-55,940 resulted in an inhibition of forskolin-stimulated cAMP accumulation. Similar results were observed using NG108-15 cells (data not shown). Panel B shows the effect of CP-55,940 on forskolin-stimulated (10 μM) cAMP accumulation in human trabecular meshwork cells. Surprisingly, CP-55,940 significantly stimulated cAMP above forskolin-stimulated levels in both trabecular meshwork and ciliary process cells ($n = 13$); similar effects were observed using WIN55212-2 (1 μM, data not shown). When used alone, without forskolin, CP-55,940 resulted in a 2.3 (±0.3)-fold and 1.7 (±0.3)-fold increase in cAMP accumulation in human trabecular meshwork ($n = 7$, $p < 0.01$) and ciliary process cells ($n = 6$, $p < 0.05$), respectively. Similar effects were observed using WIN55212. For example, WIN5512-2 resulted in a 2.8 (±0.6, $p < 0.01$) and 1.4 (±0.4, n.s.)-fold stimulation of cAMP accumulation in human trabecular meshwork and ciliary process cells, respectively. Stimulation of cAMP accumulation by CP-55,940 in trabecular meshwork cells was blocked partially following co-incubation with the CB_1-specific antagonist, SR141716A (1 μM, $p = 0.10$, panel C). Similar effects of SR141716A were observed using ciliary process cells (data not shown).

3.5. Tissue measurement of anandamide

Strips of trabecular meshwork tissue, tips of ciliary process tissue and whole neurosensory retinas were dissected from three human cadaveric eyes and snap-frozen in liquid nitrogen. Tissues were prepared for LC/MS/MS as described in Materials and methods and analyzed for the presence of anandamide. Table 1 shows a summary of our findings. Anandamide was detected in all three tissues in picomoles per gram of tissue, with ciliary process tissues having a 10-fold higher concentration than either trabecular meshwork or neurosensory retinal tissues.

4. Discussion

Using a different anti-CB_1 antibody preparation, the present study confirms the localization of cannabinoid CB_1 receptor protein to the human ciliary process and trabecular meshwork (Straiker et al., 1999a). The present study extends this finding and shows for the first time *both* messenger RNA and receptor protein in both ciliary process and trabecular meshwork from two different species, bovine and human. Furthermore, we provide the first demonstration of functional cannabinoid CB_1 receptors from these tissues that both activates heterotrimeric G-proteins and stimulates cAMP accumulation. Lastly, we present data that for the first time shows the presence of the endogenous ligand, anandamide, in ciliary process, trabecular meshwork and neurosensory retinal tissues.

In normal human eyes, the ciliary process secretes all of the aqueous humor into the eye and the trabecular meshwork drains more than 90% of aqueous humor (Epstein et al., 1997). The remaining aqueous humor exits the eye by way of the ciliary muscle and the uveoscleral outflow pathway. Cannabinoids may interact with receptors in the ciliary muscle to increase uveoscleral outflow and to lower intraocular pressure similar to that of prostanoid compounds such as latanoprost. However, the present study focused upon the two structures, ciliary process and trabecular meshwork which are primarily responsible for maintenance of normal intraocular pressure. In fact, we used two dissection techniques to isolate ciliary process and trabecular meshwork tissues that minimized contamination from unwanted cell neighbors such as ciliary muscle. For example, the tips of the ciliary process were harvested to maximize inclusion of ciliary epithelial cells, and the exclusion of ciliary muscle and vascular endothelial cells (Anthony et al., 2000). The blunt dissection method used for trabecular meshwork isolations, favor the inclusion of trabecular meshwork and inner wall cells of Schlemm's canal and exclude ciliary muscle, corneal endothelium and scleral fibroblasts (Stamer et al., 1995b).

We provide three lines of evidence using different methods that indicate low expression levels of CB_1 receptors in both the ciliary process and trabecular meshwork. First, amplification of cDNA corresponding to CB_1 messenger RNA in trabecular meshwork preparations from both cadaveric tissue and cells, was inconsistent and required 40 PCR cycles for visualization. Second, standard radioligand binding techniques using two radioligands and various protein concentrations were unsuccessful at detecting cannabinoid CB_1 receptor protein in membranes prepared from ciliary process and trabecular meshwork. Lastly, detection of functional cannabinoid CB_1 receptors using a [^{35}S]GTPγS assay required significant optimization; namely using permeabilized whole ciliary process and trabecular meshwork cells rather than membrane preparations. Detection of cannabinoid receptors by the functional [^{35}S]GTPγS assay and not by radioligand binding assays may relate to the greater specific activity of [^{35}S] compared to [^{3}H] and/or the amplification that occurs upon stimulation of a single receptor and activation of multiple G-proteins. For example, activation of a single receptor may stimulate as many as 10 G-proteins during the course of the assay.

The particular complement of G-proteins in ciliary process and trabecular meshwork cells may explain the inability of cannabinoid ligands to stimulate [^{35}S]GTPγS binding in membrane preparations. For example, the G-protein, α-transducin in a number of cells, including retinal photoreceptors, is not palmitoylated on its *N*-terminus and thus weakly associates with the plasma membrane and is subsequently lost during the preparation of membranes from cells (Chabre and Deterre, 1990; Varga et al., 2000). Thus, similar to the results presented here, intact and permeabilized cells were required to detect agonist stimulated [^{35}S]GTPγS binding in CHO cells expressing α-transducin ($G_{\alpha t1}$) (Varga et al., 2000). These data indicate that cannabinoid CB_1 receptors in trabecular meshwork and ciliary process cells are coupled to a G-protein, like α-transducin, that weakly associates with the plasma membrane.

While cannabinoid agonists decrease intraocular pressure in a manner that is reversed upon pretreatment with the specific cannabinoid CB_1 receptor antagonist, SR141716A, treatment of animals with SR141716A alone resulted in increased intraocular pressure (Pate et al., 1998). Two possibilities may explain these results. First, SR141716A acted as an inverse agonist and inhibited constitutive activation of G-proteins by receptor. Second, SR141716A blocked the interaction of endogenous cannabinoids with CB_1 receptors. Our data support the later possibility. That is, SR141716A acts as a cannabinoid CB_1-receptor antagonist since compared to basal, we found no decrease in [^{35}S]-GTPγS binding or cAMP accumulation upon treatment with SR141716A alone. Further, the endogenous cannabinoid, anandamide, was detected at significant levels by LC/MS/MS in ciliary process and trabecular meshwork tissues.

In general, activation of cannabinoid CB_1 receptors results in the inhibition of forskolin-stimulated cAMP accumulation. In this case, the inhibition of adenylyl cyclase results from a preferential coupling of cannabinoid CB_1 receptors to a pertussis toxin-sensitive Gi/Go, however, under certain situations cannabinoid CB_1 receptors can couple to Gs and stimulate cAMP accumulation. For example, treatment of striatal neurons with agonists for cannabinoid CB_1 and dopamine D_2 receptors resulted in the stimulation of cAMP accumulation (Glass and Felder, 1997). Data in the present study provides the first demonstration of preferential coupling of endogenous cannabinoid CB_1 receptors to Gs in cells not pretreated or co-stimulated. These data implicate the specific involvement of trabecular meshwork cells in the intraocular pressure-lowering effect of cannabinoids and resemble effects observed with epinephrine. Accordingly, activation of β_2-adrenoreceptors by epinephrine in the trabecular meshwork dominates over β_2-adrenoreceptor activation in the ciliary process, resulting in increased aqueous outflow and depression of intraocular pressure (Kaufman and Barany, 1981).

While the intraocular pressure-lowering effects of anandamide are well documented (Mikawa et al., 1997; Pate et al., 1996), we present the first demonstration of not only anandamide in the two tissues responsible for intraocular pressure, but in the neurosensory retina. There have been many indications for the presence of anandamide in the retina, including its synthetic precursor, *N*-arachidonylphophatidylethanolamine, as well as activities for its specific synthetase and hydrolase (Bisogno et al., 1999; Matsuda et al., 1997). However, anandamide has eluded detection in the vertebrate retina before the present study (Straiker et al., 1999b).

Cannabinoids represent a novel set of anti-glaucoma agents. The intraocular pressure-lowering effects of at least four chemical classes of cannabinoid compounds appear to interact with receptors in the eye of the cannabinoid CB_1 type. The present study presents data that is consistent with this idea and specifically implicates cannabinoid CB_1 receptors in the trabecular meshwork as targets responsible for the intraocular pressure-lowering effects of these exogenous cannabinoids and regulation of intraocular pressure by endogenous cannabinoids in vivo.

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