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Interactive report

Cannabinoid receptor CB_1 mRNA is highly expressed in the rat ciliary body: implications for the antiglaucoma properties of marihuana [1]

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Abstract

We used RT-PCR to measure relative differences in cannabinoid receptor (CB) mRNAs in the rat eye, comparing CB_1 or CB_2 transcripts to that of the normalizing reference gene β_2 microglobulin (β_2m). Significantly higher levels of CB_1 mRNA levels were found in the ciliary body ($0.84 \pm 0.05\%$ of β_2m) than in the iris, ($0.34 \pm 0.04\%$ of β_2m), retina ($0.07 \pm 0.005\%$ of β_2m) and choroid ($0.06 \pm 0.005\%$ of β_2m). CB_2 mRNA was undetectable. This expression pattern supports a specific role for the CB_1 receptor in controlling intraocular pressure, helping to explain the antiglaucoma property of cannabinoids.

Keywords: Glaucoma; THC; Gene expression; RT-PCR; CB; Eye

1. Introduction

Cannabinoids are known to exert various actions of potential medical interest, and several studies exploring their therapeutic effects on the eye have been published [1–6]. They have failed, however, to provide a clear explanation of the cellular and molecular mechanisms involved. Controversy still exists about cannabinoid gene [7,19] and protein [4] expression in the eye, and whether or not the effects of cannabinoids on intraocular pressure are directly mediated by cannabinoid receptors or through other transmitter systems [5,6].

The recent cloning and characterization of central cannabinoid (CB_1) [8,9] and peripheral (CB_2) [10] receptors allows the employment of sensitive techniques such as reverse transcription-polymerase chain reaction (RT-PCR) for the measurement and comparison of their mRNA levels. This method revealed the expression of CB_1, not only in the central nervous system but also, to a lesser extent, in several peripheral tissues such as the heart, lung, spleen and tonsils [7]. Conversely, CB_2 transcripts, which are not expressed in the brain, were found to be abundant only in the immune tissue [7]. In humans, CB_1 and CB_2 mRNA are undetectable in the retina [7], while their expression in any of the eye's other structures was not explored.

The present study was undertaken in order to determine whether, and where, cannabinoid receptor mRNAs are expressed in the rat eye. For this purpose we used a RT-PCR method previously employed to measure and compare cannabinoid receptor mRNA levels in human tissues [7].

2. Materials and methods

2.1. Animal experiments

Twenty Long–Evans rats were purchased from Charles River (Como, Italy). Animals were housed three or four per cage and given free access to food and water under controlled conditions of temperature (25°C) and humidity (65%), with a 12 h light/12 h darkness cycle. In order to minimize pain and discomfort for the animals, all the experiments were carried out in accordance with the European Community's Council Directive of 24 November 1986 (86/609/EEC).

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The rats were sacrificed by decapitation. The liver was used as negative control, while the frontal cortex and spleen were used as positive controls for CB_1 and CB_2 expression, respectively. The eye in toto was enucleated and then further dissected under a 100 × stereomicroscope (Nikon, Japan) to remove the cornea, iris, ciliary body, sclera, retina, choroid and the distal part of the optic nerve.

Total RNA was extracted by the Ultraspec® (Cinna/Biotecx, Houston, TX, USA) method, which consists of a further modification of the single step procedure of Chomczynski and Sacchi [11]. RNA concentration and purity were estimated by absorbance (A) at $\lambda = 260$ and 280 nm and gel electrophoresis, respectively. RNA preparations with an A_{260}/A_{280} ratio below 1.9–2.0 were excluded.

2.2. DNAseI treatment

RNA/DNA-containing samples were subjected to DNAseI (Fluka, Milan, Italy) treatment in a series of experiments performed in order to exclude any trace of genomic contamination from preparations. All RNA samples were always subjected to a direct PCR amplification, indicated as non-RT control.

2.3. cDNA synthesis

Three μg of RNA were reverse transcribed using the Promega-Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Briefly, the conversion of RNA into cDNA was carried out in a final volume of 40 μl, containing 5 mM $MgCl_2$, 10 mM Tris HCl (pH 8.8 at 25°C), 50 mM KCl, 0.1% Triton X-100, 1 mM of each dNTP, 2.5 units RNAsin, 15 U AMV reverse transcriptase and 0.5 μg oligo (dT)15 primer. After 10 min at room temperature and 60 min at 42°C, the reverse transcriptase was inactivated by heating the reaction to 99°C for 5 min. Synthesis of cDNA was monitored by determining the incorporation of [α-^{32}P]dCTP (Amersham, Milan, Italy), and cDNA was adjusted to 1 ng/μl and stored at −80°C in a Tris/EDTA buffer (5 mM Tris/HCl, pH 7.6, 0.5 mM EDTA) until use.

2.4. PCR conditions

To detect the amount of CB_1 or CB_2 cannabinoid receptor mRNA, we employed a non-multiplexed PCR reaction which used an endogenous sequence, corresponding to the rat β_2 microglobulin (β_2m), as an internal standard. β_2m is a ubiquitous protein whose expression represents a constant percentage of the cytosolic protein content of most eukaryotic tissues. PCR reaction was performed at a final concentration of 0.5 ng cDNA in 50 μl PCR buffer (20 mM Tris/HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$ and 0.01% gelatin), 50 μM dNTP, 3 mM $MgCl_2$, 0.25 μM each of the 5′ and 3′ primers, and 0.025 U/μl Ampli Taq^r DNA polymerase (Perkin–Elmer/Cetus). The mixture was amplified in a DNA Thermal Cycler (mod. 2400 Perkin–Elmer/Cetus). The amplification profile program published for human CB receptors was used [7]. In summary, each cycle of PCR included: 20 s of denaturation at 95°C, 30 s of primer annealing at 60°, and 20 s of extension at 75°C, for 30 cycles. After the last cycle, the reactions were incubated at 72°C for an additional 7 min extension of the PCR products. Each PCR assay was carried out in triplicate. The primers used were: CB_1 sense primer 5′-catcatcatccacacgtcag-3′ and CB_1 antisense primer 5′-atgctgttgtctagaggctg-3′ (position 1103–1122 and 1412–1432, respectively, from Genbank X55812); CB_2 sense primer 5′-tttcccactgatccctaacg and CB_2 antisense primer 5′-agttaacaaggcacagcatg-3′ (position 1092–1100 and 1391–1410, respectively, from Genbank U21681); β_2m sense primer atctttctggtgcttgtctc and β_2m antisense primer 5′-agtgtgagccaggatgtag-3′ (position 28–48 and 253–271, respectively, from Genbank RNB2MR). To avoid self- or cross-homologies between primers, all oligonucleotides were designed and checked with the aid of a specific software program (Primer-Detective, Clontech Lab., Palo Alto, CA, USA). The expected sizes of the amplicons were 329 bp and 328 bp for CB_1 and CB_2, respectively, and 243 bp for β_2m. Relative comparison of cannabinoid receptor expression levels was performed in the exponential phase of amplification in which the amount of PCR product is proportional to the initial amount of template [12,13].

2.5. Analysis of PCR products

After amplification, 25 μl of each reaction was directly subjected to gel electrophoresis on 1.8% agarose stained with ethidium bromide. Gels were visualized on a UV Transilluminator (UVP, Upland, CA, USA). Image grabbing was achieved by a Sony XC-77CE CCD video camera module (Sony, Japan), connected to an MV-LC real time frame grabber acquisition board (Matrix Vision GmbH, Oppenweiler, Germany). Images were processed by Gel-Pro Plus, an image analysis package for RT-PCR gel analysis (Media Cybernectis, Silver Spring, MD, USA).

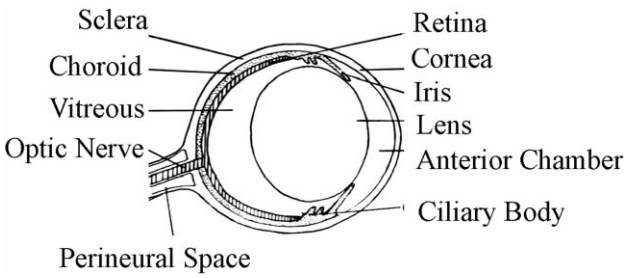

Fig. 1. Diagrammatic cross section of a rat eye indicating the subregions dissected (Adapted from Ref. [26]).

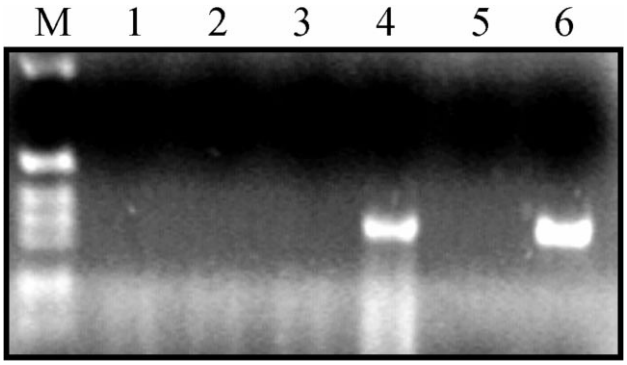

Fig. 2. Direct PCR of CB_1 expression on blood total RNA before (lane 1) and after (lane 2) 30 min of DNAseI treatment or from 1 ng. Blood cDNA (lane 3); on frontal cortex total RNA before (lane 4) and after (lane 5) DNAseI or from 1 ng of frontal cortex cDNA.

To correct for any variation in the RNA content and cDNA synthesis in the different preparations, each sample was normalized on the basis of the β_2m house-keeping gene content which was also evaluated, in parallel, in the exponential range. For comparative purposes, the CB_1 or CB_2 mRNA contents were expressed relative to the β_2m RNA content, in the same sample. Amplification efficiencies close to 70%, which were identical for the different sets of primers used and for the various samples analyzed (data not shown), made the normalization possible.

3. Results

3.1. Eye preparation

In a preliminary series of experiments the rat's eye was studied in toto, after enucleation and after the removal of the vitreous and of the optic nerve from the optic pole. Since RT-PCR experiments demonstrated positive expression of the CB_1, but not CB_2, receptors, the anatomical regions of the rat's eye were further dissected, under a 100× stereomicroscope. The sclera, cornea, lens, ciliary body with iris, vitreous, terminal part of the optic nerve, retina and choroid were separated (Fig. 1) and their mRNA purified.

3.2. DNA and blood contamination

The intronless nature of the CB_1 gene makes it particularly susceptible to genomic DNA contamination, which is also an important issue even when PCR primers spanning splice junctions are used (in other genes), because the precise quantification of pure RNA is jeopardized by the presence of DNA. A DNAseI treatment is therefore mandatory. Compare lane 4 in Fig. 2 demonstrating the CB_1 signal from direct PCR on total RNA (no RT performed) and showing DNA as a genomic smear, with a similar sample after DNAseI treatment (lane 5). A second problem we anticipated was due to possible blood contamination, since PCR has been known to show CB_1 as well as CB_2 expression from lymphocytes [7,12]. However, when RNA was extracted, in the same way as for the other tissues, from 1 ml of untreated peripheral blood (with no previous corpuscolate cell purification), CB_1 or CB_2 (not shown) expression was not detected in either RNA (Fig. 2, lane 1 and 2) or c-DNA (Fig. 2, lane 3).

3.3. Expression of CB mRNA in the eye

The specificity of amplification for CB_1 was primarily established using DNA from plasmids which only express CB_1 (Fig. 3, lane 18). The absence of contaminating genomic DNA in cDNA samples was assessed by a direct amplification on RNA samples, run in parallel for each experiment (Fig. 3, lanes 2, 5, 7, 10, 12 and 15). The expression levels of CB_1 and β_2m genes in each sample was determined by relative measurements of mRNA-derived cDNA by RT-PCR.

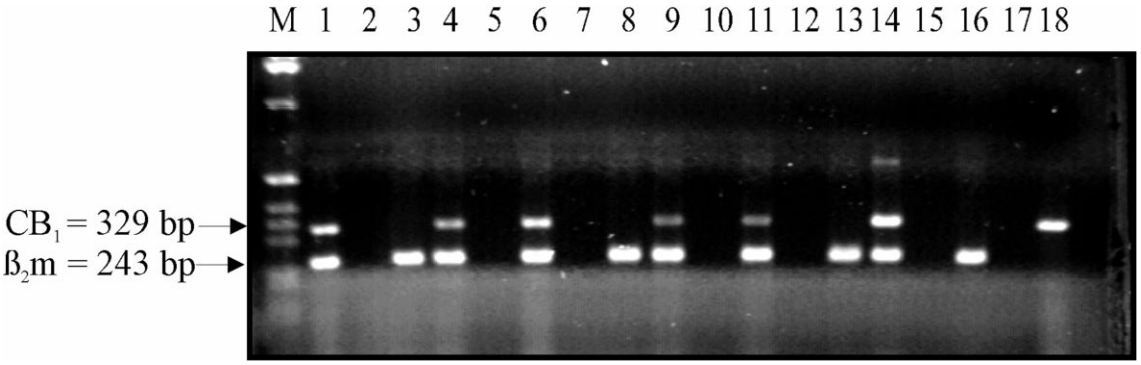

Fig. 3. Single gel RT-PCR of CB_1 and β_2m mRNA from the rat eye. M indicates molecular weight marker. Eye in toto (lane 1); Direct PCR on eye in toto RNA (lane 2); cornea (lane 3); iris (lane 4); iris RNA (lane 5); ciliary body (lane 6); ciliary body RNA (lane 7); sclera (lane 8); retina (lane 9); retina RNA (lane 10); choroid (lane 11); choroid RNA (lane 12); optic nerve (lane 13); frontal cortex (lane 14); frontal cortex RNA (lane 15); liver (lane 16); negative control = no primers on CB_1 plasmid DNA(lane 17); positive control = 1 ng of CB_1 plasmid DNA (lane 18).

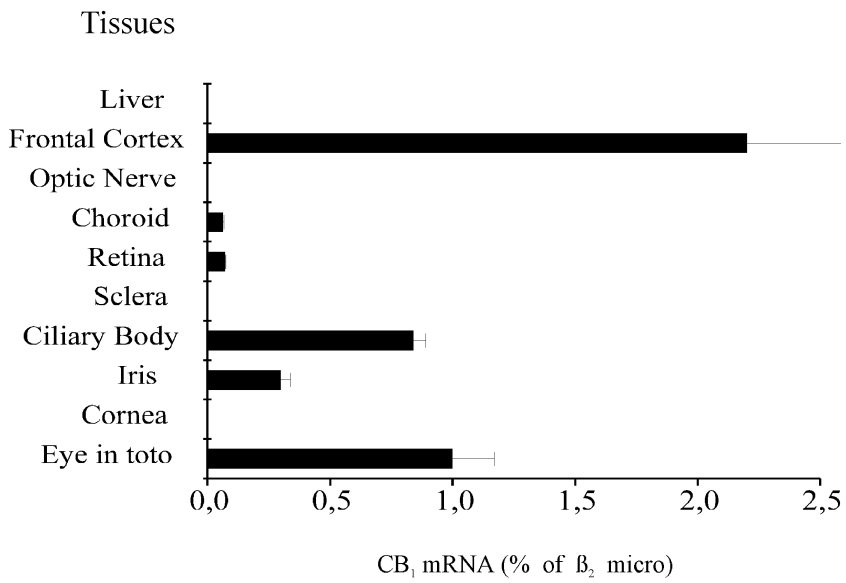

Fig. 4. Relative differences of CB_1 transcripts in the rat eye, frontal cortex and liver (as positive and negative control, respectively). The level of mRNA in the eye in toto, cornea, iris, ciliary body, sclera, retina, choroid, the distal part of the optic nerve and the frontal cortex was compared by RT-PCR. CB_1 mRNA content was normalized with that of β_2m and expressed relative to the β_2m mRNA level ($n = 6 \pm$ SEM).

3.4. Tissue pattern of distribution

The mRNA levels of CB_1 were expressed relative to the β_2m mRNA, thus allowing the comparison of CB_1 with β_2m. CB_1 expression was found in the undissected eye ($1.0 \pm 0.17\%$ of β_2m) at levels comparable to that of the human brain in toto [7]. Extending the analysis to the complete range of tissues dissected (Fig. 4), we found a much higher level in the ciliary body ($0.84 \pm 0.05\%$ of β_2m) and iris ($0.34 \pm 0.04\%$ of β_2m), when compared to the retina ($0.07 \pm 0.005\%$ of β_2m) and choroid ($0.06 \pm 0.005\%$ of β_2m) where CB_1 mRNA levels were almost undetectable; in all the other eye structures, CB_1 transcripts were absent. CB_2 transcripts were not detectable in any of the rat eye structures examined, but CB_2 primers were fully functional, and the signal produced by them was clearly distinguishable from that of CB_1 as shown when RT-PCR was performed on mRNA from the spleen (Fig. 5). Interestingly, the levels of CB_1 expression in the rat frontal cortex ($2.2 \pm 0.5\%$ of β_2m) were comparable to that of the human cortex [7], thus emphasizing the requirement for normalization of the samples with the housekeeping β_2m gene for accurate quantifications.

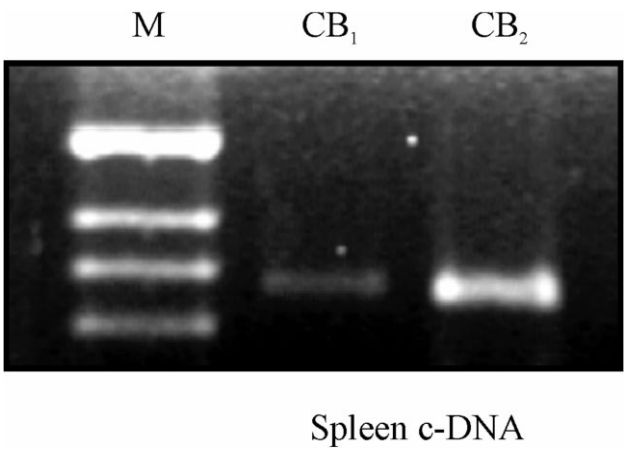

Fig. 5. RT-PCR of CB_1 (329 bp) and CB_2 (328 bp) mRNA from rat spleen, run on a high resolution 3% Nu sieve GTG agarose gel (FMC, BioProducts. Rockland, USA). M indicates molecular weight marker.

4. Discussion

Some clinical [1,2] and preclinical [3] data suggest that cannabinoids could represent a new class of antiglaucoma agents. Research interest in this application remains, in spite of the controversy about the efficacy of cannabinoids in clinical ophthalmology [2,5,6], because it may help in understanding the physiology of intraocular pressure control. In topical administration, when the lipid solubility of Delta-9-tetrahydrocannabinol (Δ9-THC) was overcome by the use of mineral oil as the vehicle for its instillation, the degree of lowering of the intraocular pressure was at least as great as that of pilocarpine, and the duration of the effect was often longer [3].

Recently, the role of CB_1 receptors in decreasing intraocular pressure in normotensive rabbits after a topical [5] or systemic [14] administration of the endogenous ligand anandamide was brought into question by the lack of action of WIN-55212-2, a compound with a great affinity for the CB_1 receptor [6].

We found that the rat's eye in toto is rich in CB_1 transcripts up to a level comparable to that of the human brain [7]; when the anatomical structures of the rat's eye

are further dissected and analyzed, the predominant CB_1 mRNA is detected in the anterior chamber and particularly in the ciliary body and iris, which express ten times more CB_1 mRNA than the retina and about as much as the human pituitary [7].

A most remarkable finding was that the ratio of CB_1 to β_2m in the eye in toto was equal to or greater than the ratio in the ciliary body, and significantly greater than that in the retina, which comprises the vast majority of cells (and therefore RNA/DNA) in the eye. One would expect the ratio of CB_1 to β_2m to be significantly less in the eye in toto than in the regions having the highest ratio of those two mRNAs (due to the dilution of the CB_1 mRNA by all the regions lacking that message). This finding could be due to several factors: (a) the dissection of the eye in toto is much faster and the risk of degradation is lower; (b) the DNAseI treatment — which must always be performed — causes a loss of RNA in the eye in toto which is less than that in the microregions dissected. In fact, the eye in toto contains between 25 and 30 μg of RNA/DNA before the DNAseI treatment, and the losses after DNAseI treatment are in the range of 40–50%; while in the microdissected regions RNA/DNA starting amounts are between 4 and 8 μg with losses of about 70–85%, necessitating frequent tissue pooling to reach the desired 3 μg necessary for the RT reaction.

The higher level of CB_1 mRNA in the ciliary body supports the demonstration that Δ9-THC is mainly a vasodilator of the efferent blood vessels of the anterior uvea, by inhibiting the sympathetic tone, since both α- and β-adrenergic antagonists reduce the Δ9-THC-induced decrease in intraocular pressure by approximately 50% [15]. This vasodilatation decreases capillary pressure within the ciliary body which is responsible for the fall in intraocular pressure [3]. These functional effects, confined to the anterior eye segment, are thought to be mediated by a specific receptor mechanism that has, however, never been identified. Since endothelial and vascular smooth muscle cells have been recently shown to be involved in the improved microcirculation obtained after localized vasodilation [16] produced by macrophage-derived anandamide [17], this mechanism of action could also be responsible for the CB_1 receptor mediated effect in the ciliary body.

We found an extremely low level of expression of the CB_1 gene in the rat's retina, but, in contrast to what is reported for humans [7], it is clearly detectable. This is probably due to the fact that we processed the tissue and extracted the RNA immediately, thereby minimizing the degradation of the nucleic acid, which is a crucial point in the extraction of such small tissues.

The level of expression in the rat's retina is comparable to that of other human tissues such as the testis, ovary, prostate and uterus, for which CB_1 mRNA represents less than 0.08% of β_2m [7]. Very recently, CB_1 receptor mRNA has been shown to be present in the retina of rat embryos, suggesting the possibility that cannabinoids could interact with receptors within the retina during development [18]. In humans prenatal exposure to marijuana decreases visual perception [19], while in animals it disrupts the habituation and reactivity to different illumination conditions [20]. Ocular expression of CB_1 is also in line with two recent reports on non-rat species. One showed that neurotransmitter release from dopaminergic neurons of the guinea-pig retina, most likely from the dendrites of amacrine cells, is modulated by inhibitory cannabinoid receptors of the CB_1 subtype, which seem to be tonically activated by endocannabinoids [21]. The other report found enzyme activity of both anandamide synthase and hydrolase in porcine ocular tissue, suggesting a physiological role for anandamide in the eye [22] mediated by a CB_1 receptor subtype.

Even if the data provided here strongly suggest the possibility of a direct CB_1 effect on the regulation of intraocular pressure, we cannot clearly conclude that the effects are truly mediated by CB_1 receptors, until studies employing now-available selective CB_1 antibodies [23,24] are performed at the level of the eye. This would help in solving the problem of CB_1 specific binding in tissues rich in fat, such as the eye, and in excluding possible high-affinity non-receptor components of binding which can be present in the eye structures (i.e., myelin basic protein [25]). Such an approach would also address concerns regarding the susceptibility of different cell types to the deposition of high levels of cannabinoids, and cell membrane perturbation as a result of these drugs. In Sprague–Dawley rats, studies at the protein level carried out by autoradiography of [^{3}H]CP-55940, a synthetic radiolabelled cannabinoid, showed high levels of nonspecific binding in the visual structures, therefore posing technical questions about the need to improve the detection of specific binding in certain tissues [4]. The eye is a tissue rich in fat, which makes the quantification of binding very difficult, since the sequestration of high levels of cannabinoids in lipophilic membranes may allow drug–membrane interaction of the type that occurs with high (micromolar) concentrations of drugs [4]. These reasons may help to explain the contradiction between apparent nonspecific cannabinoid binding [4] and the abundant expression of CB_1 receptor mRNAs in several tissues [7], known as target organs of the functional effects of cannabinoids [1].

The present data, demonstrating CB_1 expression in the rat's eye encourage further investigations at the pharmacological level, investigations which are currently being carried out in our laboratory.

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