European Journal of Neuroscience, Vol. 12, pp. 1123–1127, 2000 © European Neuroscience Association

SHORT COMMUNICATION
The human eye expresses high levels of CB1 cannabinoid receptor mRNA and protein

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Keywords: gene expression, glaucoma, marijuana

Abstract

We used reverse transcriptase polymerase chain reaction to detect the expression of the central and peripheral cannabinoid receptors (CB1 and CB2, respectively) mRNA, and Western blotting to show the presence of the CB1 protein in subregions of the human eye. CB2 mRNA transcripts were undetectable, while levels of CB1 mRNA were significantly expressed in the human retina (25.8 ± 2.46%), ciliary body (210 ± 11.55%) and iris (62.7 ± 5.94%) when compared with those of the normalizing reference gene β_2 microglobulin. The CB1 gene encodes a functional protein which is detected in its glycosylated (63 kDa) and unglycosylated (54 kDa) form in the same areas by a specific purified antibody raised against the amino terminus (residues 1–77) of the CB1 receptor. These results further support the proposed role of the CB1 receptor in controlling intraocular pressure, helping to explain the antiglaucoma properties of marijuana.

Introduction

The antiglaucoma properties of marijuana have been studied for almost 30 years (Hepler & Franck, 1971; Merrit, 1982), even if the mechanisms by which its constituent compounds lower intraocular pressure (IOP) were not completely clarified. Recently, the central cannabinoid receptor (CB1) was characterized and cloned in the rat (Matsuda *et al*., 1990) and human (Gerard *et al*., 1990) brain, and a putative endogenous ligand, anandamide, was also discovered, allowing studies which suggested important physiological roles for this novel neurochemical system (Devane *et al*., 1992).

The lack of knowledge of the distribution of cannabinoid receptors in the brain and peripheral organs has hampered attempts to elucidate the function of endogenous cannabinoids (Tsou *et al*., 1998). An otherwise detailed study of the autoradiographic distribution of the brain CB1 receptor did not investigate any of the eye structures (Herkenham *et al*., 1991) and, recently, the mRNA for CB1 receptors was not found in the human retina (Galiègue *et al*., 1995), most probably because the rapid degradation of the eye's nucleic acids was not considered. Other groups have demonstrated the presence of CB1 mRNA in the retina of rat embryos, suggesting important implications during development (Buckley *et al*., 1998) and, very recently, cannabinoid CB1 receptors and ligands were localized in the retina of several vertebrate species (Straiker *et al*., 1999).

We recently found (Porcella *et al*., 1998a) that the rat's eye is rich in CB1 mRNA, and that such expression predominates in the anterior chamber of the eye, particularly in the ciliary body, supporting a specific role for the CB1 receptor in controlling IOP and helping to explain the proposed therapeutic antiglaucoma potential of cannabinoids (Colasanti, 1990).

Cannabinoids have been shown to decrease IOP by either a systemic (i.e. hypotensive) or local (i.e. vascular or mechanical) action (Kaufmann, 1998). Because the existence of a cannabinoid receptor in the human eye was not proven, and also owing to the difficulties of separating the vascular effects (Randall & Kendall, 1998) from the psychoactive properties of marijuana, studies into cannabinoids as ocular hypotensive agents were limited.

Thus, the role of a specific CB1 effect through other effector systems, such as prostaglandins, adrenergic and/or cholinergic receptors and carbonic anhydrase inhibitors (Sugrue, 1997; Wallace & Alward, 1998), has been debated for a long time.

This study was undertaken to determine whether, and where, CB1 receptor mRNAs and protein are also expressed in the human eye.

Materials and methods

Human tissues

Five human eyes were removed, for traumatic or pathological reasons, from living patients. Informed written consent was obtained from all patients according to the Declaration of Helsinki (18 July 1964, see Varga, 1984). Whole eyes were bisected at the equator; the iris, the ciliary body and the retina were removed during surgery, individually divided into sterile tubes and immediately stored at –80 °C until further processing.

RNA extraction and cDNA synthesis

Total RNA was extracted by the Ultraspec® (Cinna/Biotecx, Houston, TX, USA). DNAse treatment and cDNA synthesis were performed as previously described in Porcella *et al*. (1998a).

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Received 18 November 1999, revised 14 January 2000, accepted 18 January 2000

Polymerase chain reaction (PCR) conditions

To detect the amount of CB1 or CB2 cannabinoid receptor mRNA, we employed a PCR reaction which used an endogenous sequence, corresponding to the human β_2 microglobulin (β_2m), as an internal standard. PCR was performed as previously described in Porcella *et al.* (1998a). The primers used were: CB1 sense primer 5′-CATCATCATCCACACGTCTG-3′ and CB1 antisense primer 5′-ATGCTGTTATCCAGAGGCTGC-3′ yielding a 330-bp fragment; CB2 sense 5′-TTTCCCACTGATCCCCAATG-3′ and CB2 antisense 5′-AGTTGATGAGGCACAGCATG-3′ yielding a 337-bp fragment; and β_2m sense primer 5′-CACGTCATCCAGCAGAGAATGG-3′ and β_2m antisense primer 5′-CGATCCCACTTAACTATCTTGG-3′ yielding a 259-bp fragment.

Analysis of PCR products

After amplification, 25 µL of each reaction was subjected to gel electrophoresis on a 5% acrylamide gel in 0.089 M Tris/HCl, 0.089 M borate, 0.002 M ethylenediaminetetracetic acid (EDTA), pH 8.0. Gels were visualized on a Transilluminator (UVP, Upland, CA, USA) and 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 RT-PCR gel analysis software (Media Cybernetics, Silver Spring, MD, USA). 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 CB1 mRNA contents were expressed relative to the β_2m RNA content in the same sample.

Protein extraction

Each eye tissue sample was homogenized at 4 °C by immersing a sonication probe (Vibracell, Sonics & Materials Inc. Danbury, CT, USA) for 10–15 s at 40% output in 100 µL of 20 mM HEPES buffer (pH 7.9) containing 125 mM NaCl, 5 mM MgCl, 12% glycerol, 0.2 mM EDTA, 0.1% Nonidet P-40, 5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, 0.5 µg/mL leupeptin and 0.7 µg/mL pepstatin A. The extracts were then treated as previously described (Porcella *et al.*, 1998b).

One-dimensional Western blotting

Sodium dodecyl sulphate (SDS) (final concentration, 2% SDS, 10% Glycerol, 5% β-mercaptoethanol) was added to aliquots of eye extracts containing 30 µg of total protein. Samples were then treated as described (Porcella *et al.*, 1998b) The blot was blocked with 4% nonfat dry milk and incubated with affinity-purified antibodies, diluted 1 : 500, raised against the first 77 residues of the rat CB1 receptor (a gift from Dr Ken Mackie), followed by peroxidase-labelled antirabbit antibody (1 : 1500; Amersham Life Science, Milan, Italy). Immunoreactivity was visualized by enhanced chemiluminescence. A specificity control was run by preabsorbing (1 h) and coincubating the antiserum (1 : 500) with the immunizing protein (4 µg/mL).

Analysis of immunoblots

Gels were visualized on the transilluminator and the images were processed with the aid of Western blot gel analysis software (Media Cybernetics). Each sample was measured on the basis of total absorbance in arbitrary units of optical density. For comparative purposes, the CB1 protein contents of the two different major bands migrating at 63 and 54 kDa, respectively, were expressed relative to each other.

Results

The specificity of amplification for CB1 was primarily established using DNA from plasmids which only express CB1 (data not shown), as previously reported (Porcella *et al.*, 1998a). The expression levels of CB1 and β_2m genes in each sample was determined by relative measurements of mRNA-derived cDNA by RT-PCR. The mRNA levels of CB1 were expressed relative to the β_2m mRNA, thus allowing the comparison of CB1 with β_2m (Fig. 1A and B). CB1 expression was detected in the human retina (25.8 ± 2.46%), ciliary body (210 ± 11.55%) and iris (62.7 ± 5.94%). CB2 transcripts were undetectable in any of the rat eye structures examined (data not shown).

Figure 2A shows a representative Western blotting with affinity-purified antibody raised against the N-terminus of the CB1 receptor, detecting two major bands of approximately 63 and 54 kDa which gave optical density readings (Fig. 2C) of 59 ± 6.20 and 35.30 ± 3.89, respectively, in the iris (lane 1); 156.30 ± 16.15 and 45.30 ± 5.99 in the retina (lane 2); 190.69 ± 20.34 and 113.93 ± 12.98 in the ciliary body (lane 3), where the CB1 protein was more abundant compared with the other two structures. The 63-kDa major band corresponds to

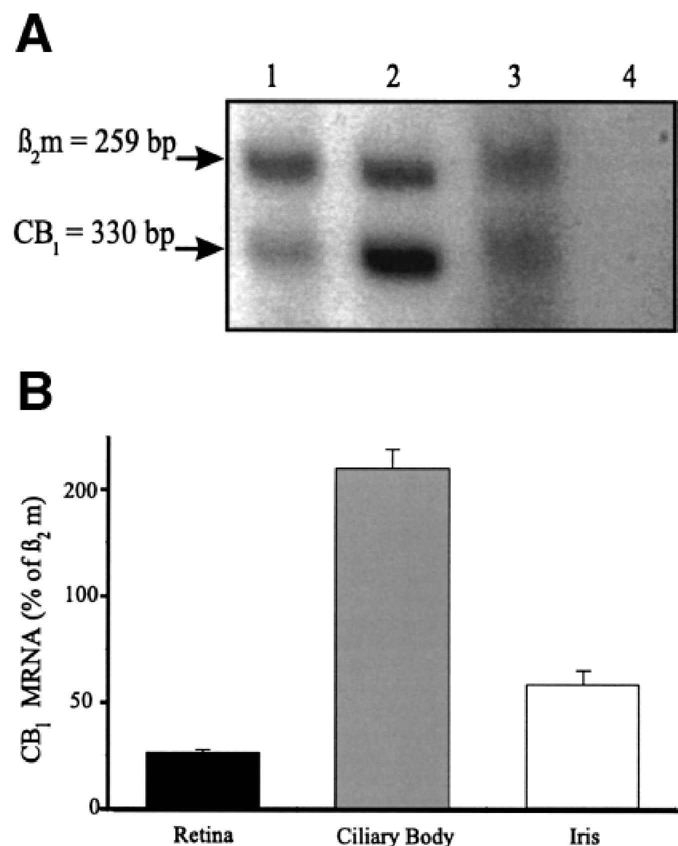

FIG. 1. (A) RT-PCR of CB1 and β_2m from human eye: retina (lane 1); ciliary body (lane 2); iris (lane 3); negative control (lane 4); run on a non-denaturing 5% polyacrylamide gel. (B) Relative differences of CB1 transcripts in the human eye. The level of mRNA in the retina (25.8 ± 2.46%), ciliary body (210 ± 11.55%) and iris (62.7 ± 5.94%) were compared by RT-PCR. CB1 mRNA content was normalized with that of β_2m and expressed relative to the β_2m mRNA level ($n = 5$; bars are SEM).

the expected molecular weight of the glycosylated form of the CB1 receptor (Matsuda *et al.*, 1990; Song & Howlett, 1995). A specificity control experiment incubating the CB1 antibody with the immunizing protein showed no immunoreactivity in any of the eye structures (Fig. 2B).

Discussion

Several preclinical (Perez-Reyes *et al.*, 1976; Pate *et al.*, 1996; Hodges *et al.*, 1997) and clinical (Hepler *et al.*, 1971; Nahas, 1984; Colasanti, 1990) data show that cannabinoids may represent a new

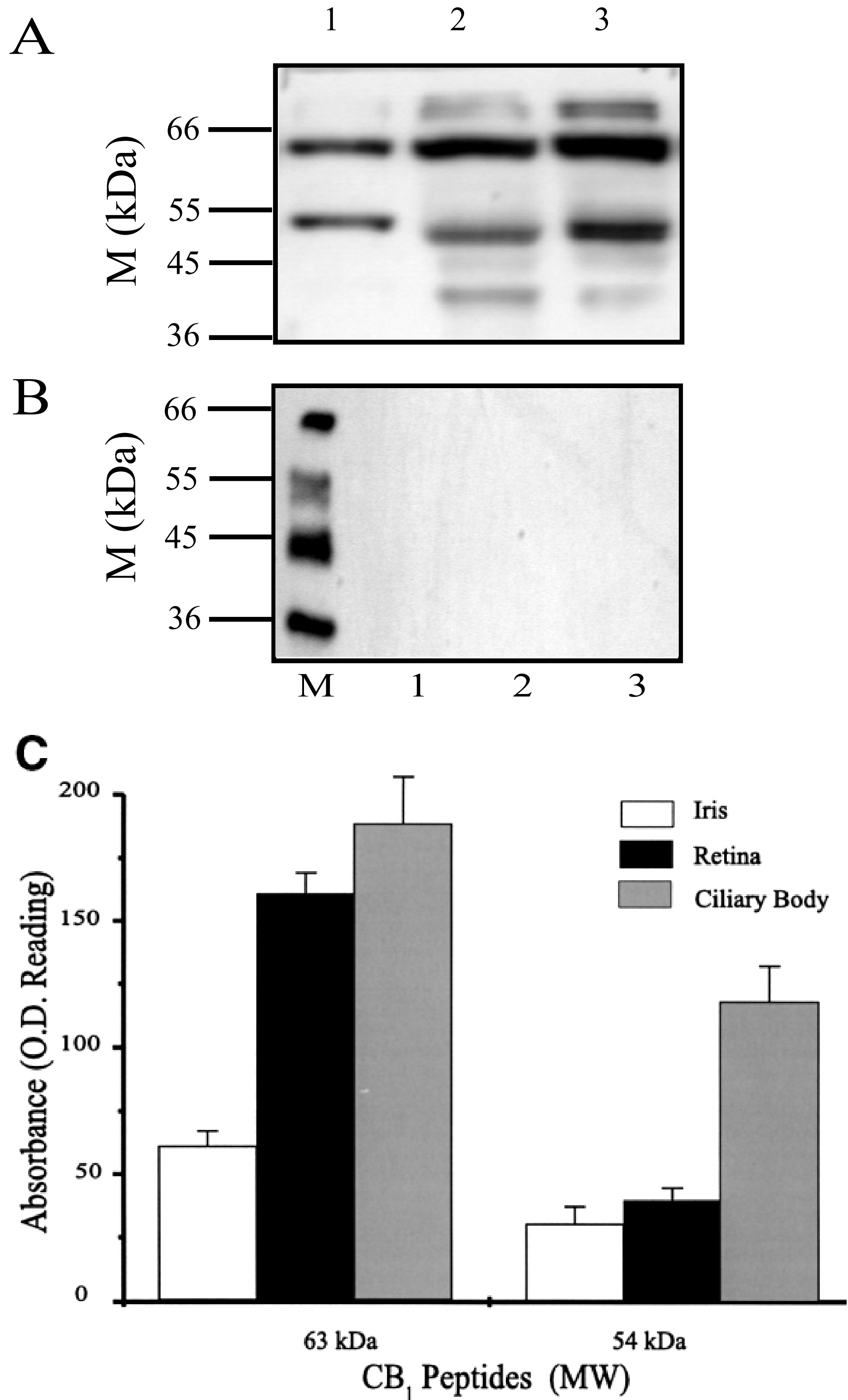

FIG. 2. (A) Western immunoblots of whole-cell extracts obtained from the human eye. Two major bands of 63 kDa and 54 kDa, respectively, were recognized in the iris (lane 1); retina (lane 2) and ciliary body (lane 3); another two bands of minor intensity were detected in the retina and ciliary body migrating at the presumptive molecular weight of 38 and 68 kDa. (B) Control experiment coincubating the antiserum with the immunizing protein, showing complete block of specific labelling. M indicates molecular weight marker. (C) Relative differences of CB1 peptides in the human eye. The level of CB1 proteins in the iris, retina and ciliary body were measured in arbitrary units of optical densities and compared ($n = 5$; bars are SEM).

class of potential antiglaucoma agents. Recently we found that the rat's eye is rich in CB1 transcripts, mostly in the ciliary body, strengthening the hypothesis of a physiological role for endogenous cannabinoids in the control of IOP (Porcella *et al*., 1998a).

The present results regarding the presence of CB1 mRNA and receptor predominantly in the human ciliary body may support the proposed role of Delta-9-tetrahydrocannabinol as a vasodilator of the efferent blood vessels of the anterior uvea (Martin, 1986). This vasodilatation is thought to decrease capillary pressure within the ciliary body, which is ultimately responsible for the fall in IOP (Hodges *et al*., 1997). We demonstrate here that these functional effects, confined to the anterior eye segment, are mediated by the CB1 receptor transcribed and translated within the same regions of the human eye.

Interestingly, the mRNA for CB1 is expressed in tissues of the human eye at relative levels which are similar to those seen in the rat, the expression predominating in the ciliary body in both species. This fact complements data on the highly conserved primary structure of the CB1 receptor, with 93% identity at the nucleic acid level and 97% homology at the amino acid level (Onanivi *et al*., 1996; Ameri, 1999) and indicates a specific role for the cannabinoid receptor in the mammalian eye (Straiker *et al*., 1999).

The CB1 gene in the human eye encodes a functional protein which is detected in its glycosylated (63 kDa) and unglycosylated (54 kDa) form in the same areas (Howlett *et al*., 1998). There are three highly conserved potential glycosylation sites in the human (and rat) CB1 protein; whether they are essential for the receptor function of the protein remains to be clearly determined (Onanivi *et al*., 1996). Our present data correspond to those of immunoblots using other antipeptide antibodies that demonstrated a molecular weight of the mature CB1 receptor of 64 kDa (Song & Howlett, 1995). Treating the receptor with endoglycosidases reduces this apparent weight to 54 kDa (Song & Howlett, 1995; Ameri, 1999). The immunoreactivity that appears at 54 kDa may represent newly synthesized receptors that may have escaped cotranslational glycosylation (Song & Howlett, 1995; Dove Pettit *et al*., 1998). The antibody we employed appears to be specific for the cannabinoid CB1 receptor, because pretreating the antibody solution with immunizing protein (gift of Dr Ken Mackie) completely blocked the specific labelling (Fig. 2B).

The 38-kDa band in the ciliary body and retina is probably a partial or degraded product of the CB1 receptor itself, and does not indicate the existence in the eye of the splice variant of the CB1 receptor isolated by Shire *et al*. (1995), denominated CB1A. Besides the molecular weight difference (the CB1A migrates at 46 kDa), the sequence analysis of this clone reveals a deletion of 167 base pairs encoding 61 amino acids near the 5′ end of the protein. As a result of this excision, the CB1A isoform lacks two of the three potential Asn-linked glycosylation sites, and the first 28 amino acids of the truncated receptor are completely different from those of CB1. Because the antibody we employed is directed against the first 77 amino acids of the CB1 receptor, this splice variant could not be detected in the present immunoblots.

Most of the mRNA for the CB1 appears to be continuously transcribed and translated in the ciliary body. Analysis of the relative percentages of the mRNA and of the peptide subpopulations suggests a condition of high RNA turnover, probably due to the decidual nature of the ciliary body epithelium.

Conversely, in the retina, a very small amount of mRNA is regularly transcribed. This corresponds to a more stable life of the retinal cells on which the CB1 is localized (ganglion cells and possibly other cell layers) (Buckley *et al*., 1998), and/or with a different stability of the receptor itself.

In the iris, the role of the CB1 mRNA seems questionable, because about 20% of the total amount detected in the eye regions examined is paralleled by a similar percentage of immature protein, implying a very low turnover and probably casting doubts on a real functional role in this tissue.

The present results do not yet produce evidence of a precise cellular localization of CB1 receptor protein. Locally produced anandamide, either in the ocular tissue (Matsuda *et al*., 1997) or macrophages, could be responsible for the vasodilatation mediated by a CB1 effect on the endothelial eye structure as recently shown in vascular smooth cells involved in microcirculation (Wagner *et al*., 1997).

Alternatively, one should consider that inhibition of presynaptic calcium channels (Twitchell *et al*., 1997) by cannabinoids, which reduces neurotransmitter release from CB1 expressed in presynaptic terminals (Schlicker *et al*., 1996), may also inhibit noradrenaline release in ocular tissues which is similar to findings in slices from the human and rat hippocampus (Schlicker *et al*., 1997); by this means they could reduce the production of aqueous humor that depends on the adrenergic tone on α_2 and β receptors localized in the ciliary processes, retina and iris (Sugrue, 1997).

In conclusion, the data provided here show that, among various classes of promising antiglaucoma agents, cannabinoid CB1 receptor agonists deserve special attention because they may serve to highlight the normal physiology of IOP control.

Acknowledgements

This work was supported by a grant from the National Research Council. Thanks are given to Dr Ken Mackie for the gift of the CB1 antibody and the immunizing protein.

Abbreviations

β_2m, beta 2 microglobulin; CB1, central cannabinoid receptors; CB2, peripheral cannabinoid receptors; EDTA, ethylenediaminetetracetic acid; IOP, intra ocular pressure; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate.

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