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UMI
Effects of Cannabinoid Agonists in the Mammalian Eye

By

Anna-Maria Szczesniak

Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy
At
Dalhousie University
Halifax, Nova Scotia
Canada

August 2005

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DEDICATION

This Thesis is dedicated for people whom I love and consider most important in my life, my husband Marek, my daughters Stephanie and Kasia, my mother Zofia and sister Jadwiga, and for those who are no longer here with me my father Józef and aunt Maria.
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ABSTRACT

Intraocular pressure (IOP) is determined by both aqueous humor (AH) production and AH outflow facility. This research examined the pharmacokinetic and pharmacodynamic profiles of liposomal Δ9-tetrahydrocannabinol (LTHC) on IOP in Brown Norway rats and New Zealand White rabbits. The IOP effects of LTHC were compared to that of other cannabinoids. The additive IOP effect of LTHC together with β-blocker (timolol) and prostaglandin analogue (latanoprost) was also investigated. The cannabinoid cellular mechanisms were studied in trabecular meshwork (TM) cells, which line the AH outflow pathway.

In rats, LTHC was given by intratracheal (i.t.) instillation or via intraperitoneal (i.p.) injection. WIN55,212-2, methanandamide (MA) and cannabinoid receptor 1 (CB1R) antagonist SR141716A were administered i.p., while timolol and latanoprost were applied topically. In rabbits, LTHC was administered i.t., i.v., or topically. IOP readings were taken every 15 minutes for 2 hours. For the pharmacokinetic experiments, topical, i.t. and i.v. LTHC was administered to rabbits. Ocular tissues were harvested and the Δ9-THC was determined by gas chromatography/mass spectrometry. The expression of CB1Rs in rat ocular tissues was confirmed with RT-PCR, Western Blot analysis and immunohistochemistry. The effects of CB1Rs activation on cell signalling pathways in TM cells were investigated with Western Blot analysis, phalloidin staining of filamentous actin, and Optical Magnetic Twisting Cytometry (OMTC) analysis.

LTHC, MA and WIN55,212-2 reduced IOP in rat eyes. These IOP-lowering effects were blocked by SR141671A. No additive effect was observed when Timolol or Latanoprost was added to LTHC. The biologically relevant amounts of Δ9-THC were detected in rabbit ocular tissues following topical, i.t. and i.v. LTHC administrations.

Cannabinoid and muscarinic agonists resulted in an increased MLC phosphorylation and changes in TM cell morphology consistent with an increase in contractility and trabecular resistance.

In conclusion, LTHC administration decreased IOP in animals and resulted in physiological levels of Δ9-THC in ocular tissues. The presence of CB1Rs in TM and modulation of TM cell contractility by cannabinoid agonists suggest a potential role for these compounds in regulation of AH outflow.
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<tr>
<td>AII</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AEA</td>
<td>arachidonylethanolamide; anandamide</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-archidonyl glycerol</td>
</tr>
<tr>
<td>AH</td>
<td>aqueous humor</td>
</tr>
<tr>
<td>α-AR</td>
<td>alpha adrenergic receptor</td>
</tr>
<tr>
<td>β-AR</td>
<td>beta adrenergic receptor</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB</td>
<td>cannabinoid</td>
</tr>
<tr>
<td>CBE</td>
<td>ciliary body epithelium</td>
</tr>
<tr>
<td>CBD</td>
<td>cannabinol</td>
</tr>
<tr>
<td>cDNA</td>
<td>single stranded DNA molecule compliment to the RNA</td>
</tr>
<tr>
<td>CE</td>
<td>cilary epithelium</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSK</td>
<td>cytoskeleton</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>D2R</td>
<td>dopamine receptor 2</td>
</tr>
<tr>
<td>DRDC</td>
<td>Defence Research and Development Canada</td>
</tr>
<tr>
<td>ED₅₀</td>
<td>dose of agonist producing 50% maximum change</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
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<tr>
<td>ET-1</td>
<td>endothelin-I</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amidohydrolase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GABA$_B$R</td>
<td>γ-aminobutyric acid receptor-B</td>
</tr>
<tr>
<td>$G_i$</td>
<td>inhibitory G protein</td>
</tr>
<tr>
<td>G protein</td>
<td>GTP-binding protein</td>
</tr>
<tr>
<td>GC/MS</td>
<td>gas-chromatography/mass spectometry</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>$G_s$</td>
<td>stimulatory G protein</td>
</tr>
<tr>
<td>HP-β-CD</td>
<td>2-hydroxypropyl-β-cyclodextrin</td>
</tr>
<tr>
<td>HTM5</td>
<td>human trabecular meshwork cell line 5</td>
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<tr>
<td>HU-210</td>
<td>6αR,10αR analog of 11-hydroxy-Δ$^8$-THC-dimethylheptyl</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
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<tr>
<td>IOP</td>
<td>intraocular pressure</td>
</tr>
<tr>
<td>ΔIOP</td>
<td>change in IOP</td>
</tr>
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<td>IP$_3$</td>
<td>D-myo-inositol 1,4,5,-triphosphate</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>i.t.</td>
<td>intratracheal</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>$K^+$</td>
<td>potassium ions</td>
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<tr>
<td>lb/in$^2$</td>
<td>pounds per square inch (pressure unit)</td>
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<tr>
<td>LTHC</td>
<td>liposomal Δ9-tetrahydrocannabinol</td>
</tr>
</tbody>
</table>
MA  Methanandamide; cannabinoid agonist
MAP kinase  mitogen activate protein kinase
mGluR  metabotropic glutamate receptor
MLC  myosin-light chain
MLCK  myosin-light chain kinase
mmHg  millimetres of mercury
M3R  muscarinic receptor 3
mRNA  messenger RNA
n  number of animals in each group
NAPE  N-arachidonoyl phosphatidylethanolamine
NPCE  non-pigmented ciliary epithelium
NMDA  N-methyl-D-asparate
NT  neurotensin
OEA  oleoylethanolamide
OMTC  optical magnetic twisting cytometry
PBS  phosphate buffer saline
PCE  pigmented ciliary epithelium
PCR  polymerase chain reaction
PE  phosphatidylethanolamine
PEA  palmitylethanolamide
PEG  polyethylene glycol
PGF  prostaglandin receptor
PG  prostaglandin

xvii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch (pressure unit)</td>
</tr>
<tr>
<td>PTY</td>
<td>purinergic receptor</td>
</tr>
<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute (unit of speed)</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium-dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SM</td>
<td>smooth muscle</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>SR</td>
<td>sacroplasmic reticulum</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>Δ8-THC</td>
<td>delta-8-tetrahydrocannabinol</td>
</tr>
<tr>
<td>Δ9-THC</td>
<td>delta-9-tetrahydrocannabinol</td>
</tr>
<tr>
<td>TM</td>
<td>trabecular meshwork</td>
</tr>
<tr>
<td>TRPV1R</td>
<td>vanilloid type 1 receptor</td>
</tr>
<tr>
<td>VSM</td>
<td>vascular smooth muscles</td>
</tr>
</tbody>
</table>
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Finally, for my late father Józef Pyka, for his love and confidence, and for teaching me that everything in life is possible, but it requires work. I miss him every day and I wish he were here to share this accomplishment.
PUBLICATIONS


CHAPTER 1

Introduction
1.1 Cannabinoid System

1.1.1 Cannabinoids

The hemp plant, *Cannabis sativa*, is one of the most widely used drugs in the world and is the unique source of a set of compounds known as cannabinoids (Iversen, 2005). *Cannabis sativa* contains over 400 different chemicals including over 60 phytocannabinoids (El Sohly, 2002). These phytocannabinoids are present in the leaves, flowers, seeds and stalks of the plant. Physiological and psychological effects associated with the use of these compounds in humans include euphoria, anxiety, impaired short term memory, relaxation, reflex tachycardia, hypothermia, anti-emetic and analgesic effects, and a reduction in intraocular pressure (IOP) (Iversen, 2005, 2003; Ashton, 2001; Hampson and Deadwyler, 1999; Thomas, 1996; Pertwee, 2001; Iversen and Chapman, 2002; Tramer *et al.*, 2001). In high doses, visual and auditory hallucinations and delusions may result (Martin and Lichtman, 1998).

Active components of *Cannabis sativa* responsible for cannabinoid effects, including Δ9-tetrahydrocannabinol (Δ9-THC), Δ8-tetrahydrocannabinol (Δ8-THC) and cannabidiol (CBD), were isolated during the 1940’s by Roger Adams (1941) and Alexander Todd (1946). Identification of the molecular structure and complete synthesis of the pure Δ9-THC (Mechoulam and Goani, 1967; Mechoulam, 1970) allowed the synthesis of cannabinoid analogs and the consequent discovery of two cannabinoid receptors, CB1 and CB2 (Devane *et al.*, 1988; Matsuda *et al.*, 1990). Although not yet identified, there is also some evidence to support the existence of one or more non-CB1/CB2 cannabinoid receptor subtypes (Breivogel *et al.*, 2001; Di Marzo *et al.*, 2000; Pertwee, 1999).
1.1.2 Cannabinoid Receptors

Cannabinoids exert their effect through specific endogenous cannabinoid receptors. CB1 cannabinoid receptors (CB1Rs) are predominantly localized to the central nervous system (CNS), with the highest densities identified in the hippocampus, hypothalamus, basal ganglia, cerebellum and the cerebral cortex. In peripheral tissues CB1Rs have been localized to adipocytes, endocrine glands, leucocytes, spleen, heart, parts of the urinary, reproductive and gastrointestinal tracts, smooth muscles, vascular endothelium and ocular tissues (Bensaid et al., 2003; Schuel et al., 1999, 2000; Straiker et al., 1999; Pertwee, 1997). The CB2 cannabinoid receptor (CB2R) shares a 44% overall nucleotide sequence identity with CB1Rs and is expressed primarily in peripheral tissues, including the immune system, bone marrow, lungs, pancreas and smooth muscle tissue, as well as in human and rat retina (Kaminski et al., 1992; Bouaboula et al., 1993; Geliègue et al., 1995; Lu et al., 2000). CB1Rs and CB2Rs belong to the seven-transmembrane domain superfamily of G protein coupled receptors (GPCRs), and mediate their biological effects via the pertussis toxin (PTX) - sensitive GTP - binding regulatory protein G\textsubscript{i/o} (Figure 1.1), however coupling to G\textsubscript{s} has also been reported (Glass and Felder, 1997). The activation of CB1R by cannabinoid agonists results in inhibition of adenyl cyclase activity (Howlet and Fleming, 1984), activation of mitogen-activated protein kinases (MAPKs) (Bouaboula et al., 1995; Rueda et al., 2000) and the opening of inwardly rectifying potassium (K\textsuperscript{+}) channels (Pertwee, 2002) (Figure 1.2). CB1Rs are also coupled to N-type and P/Q type voltage-activated calcium (Ca\textsuperscript{2+}) channels in which receptor activation causes inhibition of these channels. This effect has been suggested to
Figure 1.1  Simplified Diagram of G_\text{ai} -protein coupled cannabinoid receptor (CB1R or CB2R).
Figure 1.2  Mechanisms of action of cannabinoid agonists (i.e. Δ9-THC, WIN55,212-2) upon their binding to $G_{i/o}$-coupled CB1Rs.
result from a direct interaction of G_{i0} protein β-γ subunits with the channels (Wilson and Nicoll, 2002). The activation of CB2Rs results in both the inhibition of adenylyl cyclase and the stimulation of MAPKs. Effects of CB2R activation on ion channels activity have yet to be reported (Pertwee, 1997). Cannabinoid receptor-coupled signalling pathways can modulate synaptic communication and neuronal gene expression (for review see Piomelli, 2003).

1.1.3 CB1R Dimerization

Until recently, the accepted dogma of the scientific community was that GPCRs functioned as monomers and signalled via downstream G-proteins in a 1:1 stoichiometric ratio. However, recent experimental evidence derived from pharmacological studies, along with biochemical and biophysical techniques, indicate that GPCRs can form, as well as, function as dimmeric/ oligomeric structures. Furthermore, receptor dimerization is not only limited to homodimers (association between identical proteins) but also includes heterodimers of closely or distantly related GPCRs. (Angers et al., 2002; Eidne et al., 2002; George et al., 2002). The dimerization of GPCRs is suggested to serve various functions. Altered agonist affinity, potency and efficacy have been demonstrated in a number of GPCR systems (Rocheville et al., 2000; Jordan and Davi, 1999; Hebert et al., 1996). Furthermore, dimerization of some GPCRs seems to be mandatory for a functional unit. For example, heterologous or neuronal cells transiently expressing only the metabotropic γ-aminobutyric acid (GABA)_{B}R1 receptor were unable to form functional GABA_{B} receptors (Pin et al., 2004; Filippov et al., 2000). Heterodimerization of GABA_{B}R1 and GABA_{B}R2 is a necessary step for both cell surface expression and
functional receptor (Filippov et al., 2000; White et al., 1998; Ng et al., 1999). Detailed analysis of this phenomenon showed that the GABA$_B$R1 subunit is necessary for the binding of GABA whereas GABA$_B$R2 is essential for surface trafficking and agonist binding (White et al., 1998; Couve, 1998). The dimerization of the metabotropic glutamate receptor (mGluR) also appears to be required for full activity after agonist binding. Goudet et al., (2003) showed that the binding of the agonist to dimers composed of one wild-type receptor subunit and one mutant receptor subunit is sufficient in order to activate the receptor, however it does not produce full activity at this receptor. The dimerization of many GPCRs is suggested to occur within the endoplasmic reticulum (ER), an organelle that plays an essential role in the control of protein synthesis. For example, transforming growth factor- beta (TGF-β) receptors, TβR1 and TβR2, have been shown to form homodimer complexes within the ER (Gilboa et al., 1998) and are expressed at the cell surface in a dimeric form, which is independent of ligand binding. The dimerization of the β2-adrenergic receptors (β2AR), GABA$_B$R1 and GABA$_B$R2 is suggested to occur within the ER and is important for normal trafficking of the receptors to the plasma membrane (Salahpour et al., 2004; Pin et al., 2004). In the case of GABA$_B$R1 and GABA$_B$R2 the direct interaction of these two receptors masks the ER retention signal of GABA$_B$R1 and allows the heterodimer to reach the cell surface (Pin et al., 2004)

The CB1R has been shown to exist as either nonglycosylated or glycosylated monomers of approximately 53 and 64 kDa, respectively (Song and Howlett, 1995). There is also evidence of the existence of a CB1R aggregates with higher molecular weights (160 and 200 kDa) thereby suggesting that CB1 receptors may form trimeric or
tetrameric complexes. An antibody that specifically recognizes both the dimeric and oligomeric forms of CB1R was used to identify dimerized CB1Rs in rat hippocampal sections (Wagner-Miller et al., 2002). Whether dimerization of these receptors is mandatory for functional receptors or whether CB1R oligomers exhibit different pharmacological profiles as compared to CB1R monomers has yet to be determined.

1.1.4 Endogenous Cannabinoids

The endogenous ligands for cannabinoid receptors have been identified. The most extensively studied of these include 2-arachidonyl-glycerol (2-AG) and anandamide (arachidonylethanolamide; AEA) (Devane et al., 1992; Di Marzo et al., 1994; Mechoulam et al., 1995). In addition, noladin ether (an ether linked analogue of 2-AG) (Hanus et al., 2001), virodhamine (ester of arachidonic acid and ethanolamine) (Porter et al., 2002) and N-arachidonoyl-dopamine (NADA) (Huang et al., 2002) have also been discovered to be endogenous CB receptor agonists. Fatty acid ethanolamides that do not interact with known cannabinoid receptors include palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (Freund et al., 2003). AEA has a greater selectivity for CB1Rs than CB2Rs (Sugiura and Waku, 2000), but will also activate vanilloid type 1 receptors (TRPV1R) (Howlett et al., 2002; De Petrocellis et al., 2001). In most cells PEA is co-synthesized with AEA, and has been proposed to enhance the effects of AEA mediated at both CB1 and TRPV1 receptors (Di Marzo et al., 2002; De Petrocellis et al., 2001; Lambert and Di Marzo, 1999). 2-AG is the most specific and abundant endocannabinoid agonist and acts at both CB1Rs and CB2Rs (De Petrocellis et al., 2004; Sugiura and Waku, 2000). Unlike classical or peptide neurotransmitters, endogenous
cannabinoids are purported to be released from cells upon demand, by cleavage of membrane phospholipid precursors (Di Marzo et al., 1994). This reaction is suggested to be initiated by a rise in intracellular Ca$^{2+}$ and/or activation of neurotransmitter receptors. Anandamide is produced via hydrolysis of the phospholipid precursor N-arachidonoyl phosphatidylethanolamine (PE), and catalyzed by N-acylphosphatidy lethanolamine selective phospholipase D (NAPE-PLD) (Okomo to et al., 2004). The biosynthesis of 2-AG is mediated by the hydrolysis of membrane phospholipids through phospholipase C (PLC) which produces DAG. DAG in turn is converted to 2-AG by the action of two sn-1-selective-diacylglycerol lipases (DGL-α and DAGL-β) (Bisogno, T., et al., 2003). In vivo, endocannabinoids have a short duration of action mainly due to both their inactivation after transport into cells and rapid enzymatic hydrolysis. In cells, anandamide is hydrolysed to arachidonic acid and ethanolamine by the fatty acid amine hydrolase (FAAH) (Giuffrida et al., 2001; Di Marzo, 1998). Although FAAH also appears to contribute to the hydrolysis of 2-AG (Lang et al., 1999; Patricelli and Cravatt, 1999), the major enzyme involved in 2-AG hydrolysis is monoglycerol lipase (MAGL) (Dinh et al., 2004). The short duration of cannabinoid action limits their effect near their site of synthesis in an autocrine and/or paracrine manner (Di Marzo et al., 1999).

1.1.5 Exogenous Cannabinoids

The exogenous cannabinoids are divided, based on their chemical structures, into four groups: (1) classical cannabinoids, which include Δ9-THC, cannabidiol and cannabinoil, and the synthetic drug nabilone, (2) non-classical bicyclic cannabinoids such as CP-55,940, (3) aminoalkalindoles, including the prototype molecule WIN55,212-2,
and (4) eicosanoids, which include the endocannabinoid congener Methanandamide (MA) and related enzyme inhibitors.

In addition, selective antagonists for CB1 and CB2 receptors have been developed and include: SR141716A, AM251, AM281 and LY320135, which are CB1R- selective antagonists, CB2R- selective antagonists include SR144528 and AM630. SR141716A is the most commonly used CB1R antagonist/ inverse agonist for CB1Rs. However, a number of studies have shown that this agent induces inverse cannabinomimetic effects at sites other then CB1Rs (see review by Pertwee, 2004). For example, SR141716A antagonized capsaicin-induced and vanilloid receptor-mediated vasodilation of rat mesenteric arteries (Zygmunt et al., 1999), possibly by interaction with VR1, but not CB1Rs. SR141716A also acts as an inverse agonist in whole brain membranes obtained from CB1R°/° mice, where it decreases basal [35S]GTPγS, an effect opposite to that of AEA or WIN55,212-2 (Breivogel et al., 2001). This suggests the existence of an unknown cannabinoid receptor subtype. The neutral antagonists that have been developed more recently include, the SR141716A analogues NESS 0327 (Ruiu et al., 2003) and VCHSR (Hurst et al., 2002). In addition, two sulphonamide analogues of Δ⁸-THC, O-2654 and O-2050 have also been shown to act as CB1R antagonists (Thomas et al., 2004a; Thomas et al., 2004b). All of these agents attenuate cannabinoid effects, yet do not produce inverse agonism at tested concentrations.

1.1.6 Functions of the Cannabinoid System

Cannabinoids, acting through either CB1Rs or CB2Rs, have been shown to interact with a number of neurotransmitter and neuromodulator systems. CB1Rs in the
brain are predominantly localized in axon terminals and are involved in both short-term and long-term forms of synaptic plasticity, including depolarization-induced suppression of both excitatory and inhibitory neurotransmission (Wilson and Nicoll, 2002). For example, exogenous administration of cannabinoids disrupts behavioural learning and inhibits glutaminergic transmission and long-term potentiation (LTP) in the hippocampus. This effect suggests that cannabinoids may play a role in negative synaptic feedback mechanisms. The inhibitory effects of cannabinoids on glutaminergic neurotransmission have been suggested to be the most likely mechanisms underlying the neuroprotective action of WIN55,212-2 and Δ9-THC which have been reported to occur in cerebral ischemia animal models (Grundy, 2002; Nagayama et al., 1999) and ocular tissues (El-Remessy et al., 2003 (see section 1.5.3). These neuroprotective effects are thought to result from the inhibition of glutamate excitotoxicity leading to subsequent reduction in both intracellular Ca^{2+} and radical oxygen species. Cannabinoid agonists are also involved in cross-talk with opioid compounds in which they produce analgesic effects by either blocking pain-propagating neurotransmitters in the CNS, or by enhancing the release of endogenous opioids (Ciechewicz, 2003). High densities of both, CB1Rs and mu-opioid receptors have been reported to occur in the dorsal horn of the spinal cord (Welch and Stevens, 1992; Hohmann et al., 1999; Salio et al., 2001) as well as several areas of the brain including the caudate putamen, dorsal hippocampus and substantia niagra (Mansour et al., 1988; Mailleux and Vanderhaeghen, 1992; Rodriguez et al., 2001). This suggests co-localization of both of these receptors. The effect of the co-administration of cannabinoid agonists with morphine have been evaluated in a rat tail-flick radiant heat test and have been shown to produce synergistic analgesia, that can
be blocked by CB1R or opioid receptor antagonists (Smith et al., 1998; Cichewicz et al., 2004). Chronic administration of Δ9-THC or morphine induces cross-tolerance to the antinociceptive effect of either drug (Smith et al., 1998; Welch and Eads, 1999; Cichewicz et al., 1999). Activation of cannabinoid and opioid receptors by their agonists have also been shown to stimulate \(^{35}\text{S}\)GTP\(\gamma\)S binding in N18TG2 membranes in an additive manner (Shapira et al., 1998), an effect that suggests CB1Rs and opioid receptors use different subtypes of PTX-sensitive G-proteins. Cannabinoid agonists, but not anandamide, stimulate the release of endogenous opioids such as dynorphin A, which in turn act at both kappa and delta opioid receptors (Pugh et al., 1996; Smith et al., 1994; Welch, 1993). Furthermore, chronic treatment of rats with Δ9-THC increases the levels of gene expression of prodynorphin and proenkephalin in rat spinal cord (Corchero et al., 1997). An elevation in proenkephalin mRNA after Δ9-THC treatment has also been observed in the ventro-medial nucleus of the hypothalamus and in the periaqueductal gray (PAG) matter of rats (Manzanares et al., 1998). Both of these areas are involved in the regulation of nociception and cannabinoid-induced analgesia.

Cannabinoid systems also modulate GABA (gamma-aminobutyric acid) release in the basal ganglia and profoundly affect motor behaviour (Romero et al., 2002). The release of the endogenous cannabinoid, AEA, in basal ganglia is stimulated by membrane depolarization and dopamine D2-receptor activation (Giuffrida et al., 1999). In rats, motor behaviour stimulated with dopamine agonists was enhanced by the application of the CB1R antagonist SR141716A (Gorriti et al., 1999), and was attenuated by the inhibition of the AEA transporter AM404 (Beltramo et al., 2000). In addition, cannabinoid agonists including Δ9-THC and WIN55,212-2 inhibit ACh release in the
neocortex and hippocampus as well as contribute to the purported undesirable effects of cannabinoids on cognition. Cannabinoid antagonists such as SR141716A reverse these effects (Carta et al., 1998; Nava et al., 2001; Tzavara et al., 2003).

1.1.7 The Therapeutic Potential of Cannabinoids

Therapeutic use of cannabis or marijuana is socially, legally and medically controversial. Pharmaceutical cannabinoid compounds, including Marinol® (dronabinol, Δ9-THC) and Cesamet® (Nabilone), are used in the control of nausea and emesis produced during cancer chemotherapy. These are also used as appetite stimulants in acquired immunodeficiency syndrome (AIDS) (Beal et al., 1997; Plasse et al., 1991; Beal et al., 1995). Recently, Health Canada approved a cannabis-based spray, Sativex, for the treatment of pain associated with multiple sclerosis. The long-term use of cannabinoids is reported to be well tolerated without significant cognitive or physical impairments (Russo et al., 2002). The cannabinoid system plays an important regulatory function in many physiological processes and its manipulation may provide to be useful for future therapies. The potential therapeutic use for cannabinoids includes, among others, the alleviation of neuropathic pain, treatment of motor disorders such as Tourette’s syndrome (Muller-Vahl et al., 1998), dystonias and Parkinson’s disease (Consroe, 1998), neuroprotection in cerebral ischemia and stroke (Nagayama et al., 1999; Kim et al., 2005) and as an ocular hypotensive in patients with glaucoma (Järvinen et al., 2002) (see section 1.5.2).
1.2 Aqueous Humor and its Relation to IOP and Glaucoma

1.2.1 Glaucoma

Glaucoma is a group of diseases characterized by progressive atrophy of the optic nerve, which leads to a loss in peripheral vision and consequently may result in blindness. Glaucoma affects at least 70 million people worldwide (Lee and Higginbotham, 2005; Schappert, 1995) and its prevalence increases with age. Glaucoma is a multifactorial condition that has been associated with intraocular pressure (IOP)-dependent and non-pressure dependent factors (Brubaker, 1996). While elevated IOP is the most important risk factor for the development and/or progression of glaucomatous damage, glaucoma can also occur with normal or even below-normal IOP. Non-pressure dependent factors include systemic blood pressure abnormalities (Orgul et al., 1999; Flammer and Orgul, 1998) and abnormal effects of endogenous mediators such as glutamate, glucocorticoids, nitric oxide and endothelin (Neufeld et al., 1997; Wordinger and Clark, 1999; Vorwerk et al., 1999).

1.2.2 IOP as a Risk Factor in Glaucoma

Normal IOP in the human eye ranges between 13-19 millimetres of mercury (mmHg). An IOP above 22 mmHg is considered to be 'abnormal'. Individuals who have an IOP above this range and who develop optic neuropathy are considered glaucomatous. Those individuals with high IOP levels but who do not develop optic nerve damage on visual field examination are considered to have ocular hypertension.

IOP is determined by three factors: (1) the rate of aqueous humor (AH) production by the ciliary epithelium (CE); (2) the rate of AH outflow through the
trabecular meshwork (TM); and (3) the pressure in the episcleral veins into which Schlemm’s canal empties. Usually, an elevation in IOP is caused by an increase in resistance to AH drainage through the conventional outflow pathway.

1.2.3 Aqueous Humor

Aqueous humor (AH) is a clear fluid produced by the bilayered ciliary epithelium of the eye. It is derived from a filtrate of plasma with a protein concentration of less than 1 % (ratio of aqueous/plasma = 0.0247 g/dL) (Freddo, 1999). AH is secreted into the posterior chamber of the eye at the rate of approximately 2.5-3.0 μl per minutes (Freddo, 1999). The amount of AH in the eye ranges from 250-300 μl. Approximately 50 μl is contained in the anterior chamber with the remaining located in the posterior chamber. The rate of AH formation is modulated by an organism’s circadian rhythm, with the highest production just after awakening and the lowest production during sleep (Reiss, 1984).

AH plays an important role in the physiology of the eye. The balance between the inflow and outflow rate of AH determines IOP and maintains the cornea’s curvature thus preserving the optic properties of the eye (for review see Civian, 1998). The low protein content of the AH and its refractory index are very similar to that of the cornea. It does not reflect or refract light. AH also provides nutritional support (glucose, oxygen, electrolytes) for the avascular tissues of the eye including the cornea and crystalline lens as well as removes metabolic waste products (lactate, pyruvate, carbon dioxide) from these tissues (Scullica, 1996).
In addition, AH serves an immunoregulatory function as it delivers antibodies and drugs to avascular tissues. One of the best-studied proteins involved in the immunoregulatory processes of the anterior chamber is transforming growth factor beta (TGF-β) (Cousins et al., 1991; Jampel et al., 1990). Elevated levels of this protein have been reported in AH of patients diagnosed with primary open-angle glaucoma and it has been suggested that this growth factor may play an important role in the pathogenesis of the disease (Lutjen-Drecoll, 2005; Tripathi et al., 1994).

1.2.4 Aqueous Humor Inflow and Outflow Pathways

AH is secreted from the non-pigmented ciliary epithelium (NPCE) into the posterior chamber. It then flows between the iris and lens into the anterior chamber of the eye (Figure 1.3). The majority of AH passes through the TM, to Schlemm’s canal, leaving the eye by the episcleral and anterior ciliary veins and eventually entering the systemic circulation (Gabelt and Kaufman, 2005) (Figure 1.3).

The remaining AH is drained by the uveoscleral or via unconventional pathways, which include extracellular spaces within the ciliary muscle, suprachoroidal space and the posterior pole of the eye. The amount of AH outflow through the uveoscleral pathway at normal IOP varies significantly among species, however in the human eye it accounts for less than 10% of the total volume (Bill, 1971). Uveoscleral outflow is relatively pressure independent although it accounts for higher outflow facility with IOP elevation.
Figure 1.3 Diagram of the aqueous humor (AH) pathway through the anterior portion of the eye. The AH is produced by the ciliary body epithelium (CBE), by NPCE and PCE cells. It is released into posterior chamber of the eye and flows through the pupil into the anterior chamber. Most of the AH leaves the eye through the trabecular meshwork, into the aqueous vein. (Modified from Snell and Lemp, 1998).
1.2.5 Anatomy and Physiology of the Ciliary Body

The ciliary body (CB) of the eye is a 5-6 mm wide circular tissue, which extends from the anterior aspect of the scleral spur to the posterior aspect of the ora serrata (Figure 1.4). The CB is divided into two regions: the pars plicata and pars plana. The pars plicata, the anterior region, begins at the iris root and is characterized by finlike ciliary processes (approximately 70), which project into the posterior chamber. The pars plana, the posterior and flat inner surface of the ciliary body, lies behind the ciliary processes and stretches from the root of the iris to the ora serrata (Freddo, 2001: Civian 1998).

The CB consists of the ciliary muscles, CB epithelium (CBE) and loose, areolar connective tissue termed stroma. The ciliary muscle lies in the anterior two-thirds of the ciliary body and is firmly attached to the lens zonules (suspensory ligaments). The ciliary muscle suspends the crystalline lens and alters its shape in a process known as accommodation (Gillum, 1976). The stroma of the CB is a thin layer of tissue, which lies between the ciliary muscle and the CBE. The stroma also projects into the core of each of the ciliary processes and contains fenestrated capillaries, which provides the vascular supply.

The CBE is the only structure in the eye responsible for the production of AH. The CBE consists of two layers of epithelial cells, which are attached to one another at the apical surfaces (Figure 1.4). The layer adjacent to the CB stroma is composed of low cuboidal cells that contain pigmented granules, and this is known as the pigmented ciliary epithelium (PCE). The layer closest to the posterior chamber and facing the AH, the non-pigmented ciliary epithelium (NPCE) does not contain any melanin. Gap junctions link
Figure 1.4 Simplified diagram of the CBE. (A) Two layers of cubical cells: non-pigmented ciliary epithelial (NPCE) cells, which face the interior of the eye, and pigmented ciliary epithelial (PCE) cells, which neighbours the stroma. (B) The posterior surface of the ciliary body, which shows the ciliary processes and ciliary body. (Modified from Snell and Lemp, 1998)
adjacent cells within and between these layers and allow for direct communication
between the cells to form a functional syncytium (McLaughlin et al., 2004). AH is
produced from the plasma filtrate, with ions such as sodium and chloride being actively
transported into the PCE by electroneutral antiports and symports. From the PCE the
solute passes into the NPCE via gap junctions. Na\(^+\), K\(^+\) and Cl\(^-\) are transported across the
basolateral membrane of the NPCE via the activation of Na\(^+\) K\(^+\) ATPase and Cl\(^-\)
channels, respectively. The osmotic gradient, which is created by the movement of these
solute, draws water into the cleft.

The CBE expresses a number of different receptors and enzymes that effect AH
production. The best-examined receptors localized in this structure are the β-adrenergic
receptors (β-ARs) that are coupled to G\(\alpha\) proteins. Activation of these receptors results
in an increase in cyclic-AMP (cAMP) levels and stimulation of AH secretion. Blocking
β-ARs limits the formation of AH and is a therapeutic strategy used for the treatment of
glaucoma (Zimmerman and Kaufman, 1977; Berriospi and Leibowitz, 1982) (see section
1.4.1). In addition to β-AR, the CBE also expresses G\(\alpha\) protein coupled α\(_2\)-adrenergic
receptors (α\(_2\)-AR), A\(_1\) and A\(_3\) adenosine receptors and CB1Rs. Agonist activation of
these receptors results in the inhibition of AC and a decrease in cAMP formation. An
enzyme that plays an important role in AH production is carbonic anhydrase. This
enzyme is located within NPCE cells, at the tips of ciliary processes, and is responsible
for the catalysis of HCO\(_3^-\) in a straightforward reaction:

\[
H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad \text{Reaction 1}
\]
Blockade of carbonic anhydrase reduces the production of AH, and is used in the treatment of ocular hypertension/glaucoma (Strahlman et al., 1995; Donohue and Wilensky, 1996) (see section 1.4.1)

The CBE also synthesizes and secretes a number of neuropeptides and regulatory peptides that act in an autocrine fashion or target other ocular sites such as the trabecular meshwork, thereby modulating AH production and outflow. (Ortego et al., 1996; Ortego and Coca-Prados, 1997). The neuropeptides secreted by the CBE include neurotensin (NT), chromogranin C (Ortego et al., 1996) and atrial natriuretic peptide (ANP). The presence of receptors for these peptides, including NT receptors (NTRs) and atrial natriuretic receptors (NPR-A, NPR-B and NPR-C) has been reported in both PCE and NPCE cells (Ortego et al., 1996; Ortego and Coca-Prados, 1998). Components of the renin-angiotensin system (RAS) (renin mRNA, angiotensin converting enzyme mRNA, and angiotensinogen mRNA), which plays an important role in the control of electrolyte homeostasis, renal haemodynamics and blood pressure (Oparil and Haber, 1974) has also been identified in human CB and AH (Van Haeringen, 1996; Wagner et al., 1996). The biological effector of this system, angiotensin II (AII), has been shown to increase intracellular Ca^{2+} and subsequently increase K^{+} channel activity in human NPCE cells. The net result of AII action in these cells is a reduction in cell volume and a decrease in AH production (Culliane et al., 2002).

1.2.6 Anatomy and Physiology of the Trabecular Meshwork

The trabecular meshwork (TM) and Schlemm’s canal comprise the pathway for aqueous humor outflow. The TM is located at the angle formed by the cornea and iris
(Figure 1.5). Different cell types and distinct extracellular matrix arrangements exist in the TM outflow pathway. Specifically, the inner region of the TM, nearer the anterior chamber, is composed of trabecular beams. These are composed from elastin and collagen fibres that are covered by a single layer of flattened endothelial-like cells (Polansky and Alvarado, 1994; Potau et al., 2001). These cells lie on a well-developed basement membrane with one cell often covering more than one trabecular beam. The beams are arranged in a three-dimensional network and form irregular openings that are between 25-75 microns in diameter.

There are three outflow components of the TM. The outer corneo-scleral region adjoins the inner wall of Schlemm's canal. The juxtacanalicular (JXT) region (also termed the cribriform layer) contains several layers of cells which are immersed in extracellular material (ECM) yet do not have collagenous beams. These cells are connected with each other and to the cells of the inner wall by cellular processes. The last section of the outflow pathway, the inner wall of Schlemm's canal, is composed of a monolayer of cells that are highly connected to each other, are of endothelial morphology and are characterized by unique structures known as "giant" vacuoles (GVs) that are thought to provide resistance to AH flow (Bill, 1974).

TM cells express a range of receptors for neurotransmitters and peptides. Muscarinic (M3R) (Thieme et al., 2001), adrenergic (α2AR, β2AR) (Stamer et al., 1996; Jampel et al., 1987), purinergic (P2Y1, P2Y4, P2Y11) (Wax et al., 1989; Crosson et al., 2004), prostaglandin (PGF2α) and CB1Rs have been identified in TM cells using
Figure 1.5  Diagram of the anterior chamber of the eye, showing structures in the corneo-iridal angle. (Modified from Snell and Lemp, 1998).
immunofluorescence microscopy and functional assays (Stamer et al., 1996; Wiederholt et al., 1996; Anthony et al., 1998; Thieme et al., 2001). The stimulation of these receptors with different endogenous or exogenous agents modulates TM cell secretion of enzymes and structural ECM proteins, as well as affects ECM remodelling, cytoskeleton reorganization and reactive oxygen species scavenging (Gonzales et al., 2000). For example, stimulation of human and bovine TM cells with P2Y receptor agonists such as ATP or 2-methyl-thio-adenosine triphosphate (2-MeS-ATP) results in the mobilization of intracellular Ca$^{2+}$ and the activation of the extracellular signal regulated kinase (ERK1/2) (Crosson et al., 2004), an effect which results in an increase in TM cell contractility. In addition, the cells of the TM express a number of growth factor receptors, TGF-β being the most extensively studied (Wordinger et al., 1998; Borisuth et al., 1992). TGFβ-2 agonists increase extracellular matrix (ECM) by increasing the expression of metalloproteinases inhibitors (Alexander et al., 1998). In addition, TGF-β has been shown to decrease TM cell proliferation and motility in vitro, which in turn may decrease TM cellularity (Borisuth et al., 1992).

The TM serves a number of biological function including phagocytosis, chemotaxis and the synthesis and secretion of (ECM) proteins and enzymes that play an essential role in the architecture of the outflow pathway. Changes in both the morphology and biochemistry of the TM are strongly correlated to increased resistance to AH outflow and an increase in IOP (Rohen, 1983).
1.3 TM Cells Modulate AH Outflow

1.3.1 Regulation of Outflow Facility by TM Cells

The TM tissue is the major site for AH outflow from the human eye and is actively involved in maintaining IOP. AH outflow through the TM was previously thought to be solely controlled by the ciliary muscle with outflow resistance being reduced by ciliary muscle contraction and consequent expansion of the area for AH flow (Rohen et al., 1967). However, more recent evidence suggests that TM cells lining the passage of the AH outflow pathway also regulate resistance to AH outflow. The cytoskeleton of TM cells, in particular actin microfilaments, plays an important role in both cell shape and adhesion. Cellular contractility and relaxation as well as adhesive properties of TM cells control AH outflow in response to chemicals, hormones and neurotransmitters (Tian et al., 2000; Wiederholt et al., 2000), some of which are secreted by the ciliary epithelium and other ocular cells into the AH, with the TM cells releasing others.

Support for the modulation of TM cell shape and actin architecture by endogenous and exogenous factors (Erickson-Lamy et al., 1991) in contributing to outflow resistance comes from studies with glaucoma patients as well as in vivo and in vitro experiments. For example, endothelin 1 (ET-1), a potent vasoconstrictive peptide, is elevated in the AH of glaucomatous eyes and has also been shown to affect the structural properties of TM cells. In vitro experiments showed that in bovine TM cells, treatment with 1-100 nmol.L⁻¹ of ET-1 results in a dose-dependent increase in intracellular Ca²⁺ and an increase in cell contractility (Kageyama et al., 1996). Another agent that induces ocular hypotension or glaucoma via its effects on the TM is dexamethasone (DEX), a
topical glucocorticoid that is used in the treatment of ocular inflammation. *In vivo* and *in vitro* studies suggest that DEX treatment causes significant changes in both the morphology and function of TM cells, affecting their cytoskeletal structure, adhesion and synthesis of ECM proteins (Clark *et al.*, 2005; Clark *et al.*, 1994; Wilson *et al.*, 1993; Wordinger and Clark, 1999), as well as altering their cellular junctions (Dickerson *et al.*, 1998; Underwood *et al.*, 1999). The net effect of DEX treatment is an increased resistance to AH outflow, and the development of hypertension/glaucoma.

### 1.3.2 Mechanisms Underlying TM Cell Contractility and Relaxation

TM cells have been shown to have vascular smooth-muscle (VSM) - like properties (De Kater *et al.*, 1992). In TM, as in VSM, the regulation of cell contractility is dependent upon the intracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)] which can vary due to the activation of voltage-dependent Ca\(^{2+}\) channels or through the release of Ca\(^{2+}\) from internal stores (i.e. from the sacroplasmic reticulum (SR)). The free Ca\(^{2+}\) binds to calmodulin, forming a calcium-calmodulin complex (Ca\(^{2+}\)-CaM) which is then capable of phosphorylating myosin-light chain kinase (MLCK). MLCK phosphorylates myosin light chain protein (MLC), and allows for cross-bridge formation between the myosin heads and the actin filaments, thereby resulting in cell contraction (Hartshore *et al.*, 1998; Epstein *et al.*, 1999) (Figure 1.6).

In addition to a Ca\(^{2+}\)-dependent mechanism for TM cell contraction, TM contractility is also linked to Rho-kinase, an important downstream effector of activated Rho, a small guanosine triphosphatase (Rho-GTPase). Rho-GTPase inhibits myosin light chain phosphatase (MLCP), resulting in the accumulation of phosphorylated MLC and
Figure 1.6  Signalling pathways involved in alteration of contractility in SM and non-SM-cells.
subsequent cell contraction (Gong et al., 1996; Hirata et al., 1992) (Figure 1.6). Thus, even as Ca\(^{2+}\) levels in the cell decline and MLCK activity is decreased, the MLC phosphorylation persists. The application of pharmacological agents, such as endothelin-1, thromboxane A2 mimetic U-46619, angiotensin II, or carbachol can induce activation of Rho-GTPase and MLC phosphorylation in TM cells (Rao et al., 2005). Contraction of TM cells reduces the permeability of the TM to AH due to the fact that the size of the intercellular space is reduced. As a consequence of an increased resistance to outflow, AH volume increases, producing an increase in IOP (Sabanay et al., 2000). A number of pharmacological agents have been shown to interfere with the contractility of TM cells. For example, the serine-threonine kinase inhibitor H7 is a broad-spectrum phosphorylation inhibitor that affects various protein kinases, including protein kinase C (PKC), MLCK and Rho kinase (Hidaka et al., 1984; Chrzanowska-Wodnicka and Burridge, 1996). The addition of H7 to TM cells results in the deterioration of the actin microfilament system and perturbation of membrane anchorage.

A large increase in outflow facility is also achieved with other cytoskeletal active agents including latrunculin A (LAT-A) and cytochalasin B and D. Cytochalasin B, a fungal metabolite that aggregates actin filaments into the anterior chamber, has been shown to increase AH outflow facility up to six-fold, an effect that was independent of ciliary muscle disinsertion (Kaufman and Bárány, 1976). LAT-A is a macrolide agent, derived from the marine sponge *Latrunculia magnifica*, which sequesters monomeric actin and therefore inhibits actin assembly and disrupts the actin microfilament network. LAT-A interferes with cell contractility and adhesion and in both a dose and time-
dependent manner increases the outflow of AH, decreasing the IOP (Liu et al., 2003; Peterson et al., 1999, 2000).

Understanding the regulatory mechanisms influencing outflow resistance for aqueous humor, and therefore the possible control of IOP, may provide a potential tool for development of new agents for the treatment of glaucoma.

1.4 Pharmaceutical Management of Glaucoma

1.4.1 Current Glaucoma Treatment

IOP is the principal modifiable risk factor in glaucoma. Drugs that decrease IOP, by either decreasing AH secretion or increasing AH outflow, have been shown to slow the progression of the disease (Coleman, 2003; Pate et al., 1998; Song & Slowey, 2000; Chien et al., 2003; Mittag et al., 2004). Pharmacological agents used in the treatment of glaucoma include α-AR agonists, β-AR blockers, cholinergic and prostaglandin agonists, and carbonic anhydrase inhibitors.

β-AR blockers are the most commonly used agents for the treatment of ocular hypertension and glaucoma. The IOP-lowering effect of these agents is accomplished by the reduction of AH production due to the blockage of sympathetic output at the level of the ciliary epithelium. Two types of topical β-blockers are available for the treatment of glaucoma: nonselective blockers, which block both β1 and β2 adrenergic receptors, and β1-selective blockers. Of the nonselective β-blockers, timolol is probably the most effective agent for lowering IOP; it is used once or twice daily with relatively few ocular adverse effects.
In some patients β-blockers alone are ineffective or insufficient in reducing IOP. Therefore other agents, such as latanoprost or pilocarpine, which act at different ocular sites, are used alone or in combination with β-blockers to provide hypotensive effects. The combination of different drugs often results in additive effects, thereby leading to a higher magnitude of IOP reduction.

Prostaglandins (PGs) are autacoids that are produced by cells within the outflow pathway of the eye and have been suggested to play a role in the normal regulation of AH outflow. Latanoprost is a topical prostaglandin F2α (PGF2α) analogue, which has been used for the treatment of glaucoma and ocular hypertension (Nomura and Hashimoto, 2000). Latanoprost is activated by deesterification as it passes through the cornea, when it reaches the intraocular tissues it activates prostanoid receptors within the uveoscleral pathway, therefore resulting in the formation of cAMP and an induction of c-Fos and c-Jun expression (Schachtschabel et al., 2000). These signals lead to an elevated synthesis of matrix metalloproteinases and a reduction in extracellular matrix components within the ciliary muscle, iris root and sclera (Weinreb et al., 2002) as well as relaxation of the ciliary muscle. The consequent increase in the uveoscleral outflow facility, results in a decrease in IOP (Toris et al., 1993).

Latanoprost is administered topically once daily as a monotherapeutic agent and has been shown to be an effective agent for lowering IOP as compared to placebo treatment or other anti-glaucoma agents (Camras, 1996). Furthermore, latanoprost has been shown to produce an additional IOP-lowering effect when used in combination with the β-adrenergic blocker, timolol, or the cholinergic agonist pilocarpine (Diestelhorst et al., 1997; Diestelhorst, 2000; Diestelhorst et al., 2002).
Carbonic anhydrase inhibitors, such as dorzolamide, are the second-line treatment of glaucoma. They block the enzyme carbonic anhydrase (CA)(see section 1.2.5), and inhibit generation of $\text{HCO}_3^-$ secreted by the CE into the posterior chamber of the eye. The relatively low pH (5.65) and high viscosity of the topical preparation of dorzolamine (Trusopt®) has been shown to generate local irritation (Silver et al., 2000). A recent study by Singurdsson et al (2005) showed that dorzolamide formulated in methylated $\beta$-cyclodextrin provides a sufficient concentration of the drug in ocular tissues and may limit the irritation associated with topical application.

1.4.2 The Limitations of Glaucoma Therapies

Current pharmacological treatments of glaucoma slow down the progression of optic neuropathy but also produce side effects. For example, the ocular side effects of timolol include irritation and allergic conjunctivitis, while the systemic side effects include cardiovascular responses (i.e. bradycardia), respiratory distress (bronchospasm), depression and impotence (Demailly, 1996). In comparison, prostaglandins have relatively few systemic side effects, but their use can result in increased iris pigmentation and intraocular inflammation.

In addition to negative undesirable side effects, there are also patients who do not respond to conventional therapies. For these patient groups, it is important to develop novel pharmacological agents that may have a more favourable pharmacological profile. In order to do this, greater understanding of the pharmacology of endogenous ocular regulatory systems and the cellular mechanisms regulating inflow and outflow facilities of AH is needed.
1.5 Effects of Cannabinoids in the Eye

1.5.1 Cannabinoids in the Eye

Positive immunostaining for CB1Rs and CB2Rs, as well as the presence of CB1R mRNA in various ocular tissues implies a constitutive role for cannabinoids in different ocular functions. CB1Rs are expressed in human retina and in the anterior part of the eye including: CE, corneal epithelium and endothelium, TM, Schlemm’s canal, ciliary muscle, and in blood vessels of the ciliary body (Porcella et al., 1998, 2000; Straiker et al., 1999; Yazulla et al., 1999). The presence of CB1Rs in ocular tissues of other species has also been determined and includes rat ciliary body and retina, as well as mouse, chick, goldfish and rhesus monkey retina. The expression of CB2Rs has been localized in the retina of human and rat eyes (Lu et al., 2000). The endogenous ligands for cannabinoid receptors, AEA, 2-AG and the anandamide congener PEA have been identified in human cornea, iris, ciliary body and retina (Chen et al., 2005) in both normal and glaucomatous tissues, as well as in various ocular tissues of rat and bovine (Stamer et al., 2001; Bisogno et al., 1999; Straiker et al., 1999). Interestingly, the level of both 2-AG and PEA have been shown to be significantly decreased in glaucomatous human eyes in the ciliary epithelium, a structure which play an essential role in the regulation of AH flow (Chen et al., 2005).

Cannabinoid ligands acting through CB1Rs have been shown to decrease IOP, regulate photoreception and neurotransmission in the optic nerve and provide protection against glutamate excitotoxicity (Schlicker et al., 1996; Fan and Yazulla, 2003; Yazulla et al., 2000; Pate et al., 1998; Porcella et al., 2001). The ability of cannabinoids to
decrease IOP and provide neuroprotection may provide potential benefits in the treatment of glaucoma.

1.5.2 The IOP-Lowering Effects of Cannabinoids

Hepler and Frank (1971) first reported initial evidence of marijuana being able to exert an ocular hypotensive action. Since then, human studies using oral (Merritt et al., 1980b) and intravenous (i.v.) (Cooler and Gregg, 1977) administration of Δ9-THC, the major active constituent of marijuana, confirmed this finding. In addition, animal models using the endogenous endocannabinoid, anandamide, and synthetic cannabinoids, such as WIN55,212-2 and CP-55,940 also reported a decrease in IOP (Pate et al., 1998; Song and Slowey, 2000). A reduction in IOP was also produced by inhibition of FAAH (an enzyme which hydrolyzes AEA) (Laine et al., 2002) or by inhibition of cellular reuptake of endogenous AEA by AM404 (Laine et al., 2001).

It has been suggested that the ocular hypotensive actions of Δ9-THC and other cannabinoid drugs are regulated by the central nervous system (CNS). However, Colosanti (1986) reported that the decrease in IOP in cat eyes treated with cannabinoids applied locally is substantially lower than that of the untreated eye, thereby suggesting a local effect. Moreover, the direct administration of various cannabinoids into the cerebral ventricle of rabbits had no effect on IOP (Liu and Dacus, 1987). The high levels of CB1R expression in CBE and TM implies that cannabinoid compounds may exert their effects on AH production and/or AH outflow. In normal and glaucomatous monkey eyes, the mechanism of IOP reduction by WIN55,212-2 appears to be mediated by the reduction of AH flow (18%), with the outflow facility remaining unchanged (Chien et al.,
2003). Cannabinoid agonists may target CB1Rs expressed on efferent blood vessels and produce vasodilation - induced decrease in capillary pressure, and a consequent reduction in AH production. This effect may also be mediated by cannabinoid activation of K\(^+\) channels and Ca\(^{2+}\) influx. For example, in mesenteric arteries vasorelaxation produced by AEA was abolished by charybdotoxin (which blocks large conductance Ca\(^{2+}\)-activated K\(^+\) channels and voltage sensitive K\(^+\) channels) as well as apaminutes (which blocks small conductance Ca\(^{2+}\)-activated K\(^+\) channels) (Randall and Kendall, 1998). Finally, a recent study by Lograno and Romano (2004) showed that AEA and CP55,940 induce contractions in bovine ciliary muscle strips, an effect which can be inhibited by pre-administration with SR141716A. This contractility can also be inhibited with the phospholipase C inhibitor U73122, or potentiated by the protein kinase C activator phorbol 12,13 dibutyrate (PDBu). This suggests that cannabinoid-induced contractility in CM maybe mediated via a phospholipase C (PLC) pathway (Lograno and Romano, 2004).

1.5.3 Neuroprotective Effects of Cannabinoids in the Eye

In glaucoma, loss of vision is ultimately due to the death of retinal ganglion cells (RGCs) via apoptosis (Quigley, 1999). Apoptosis of RGCs may be multifactorial and could be due to a number of factors possibly including axon injury at the optic nerve head arising from mechanical or ischemic insults and involving glutamate receptors, or excitotoxicity due to activation of glutamate receptors (Dreyer and Lipton, 1999). Recent studies have suggested that in addition to their IOP-lowering properties cannabinoids also may play a neuroprotective role. Δ9-THC, cannabidiol (CBD), the nonpsychotropic
component of marijuana, as well as the synthetic nonpsychotropic cannabinoid agonist, HU-211 have been shown to be neuroprotective in glutamate- or NMDA-induced cell death in cell culture models and as well as after NMDA-induced retinal neurotoxicity. In cultured hippocampal neurons, CB1R mediated neuroprotection is mediated by a decrease in cAMP-dependent protein kinase A (PKA) and changes in the sensitivity of the type –II ryanodine receptors (RyRs). The net result was a reduction in $\text{Ca}^{2+}$ levels and increased survival of neurons after excitotoxic (NMDA) insult (Zhuang et al., 2005). El-Remessy et al. (2003) showed that neuroprotection after systematic administration of $\Delta^9$-THC or CBD is produced via the attenuation of an excessive formation of reactive oxygen species including peroxynitrate. This neuroprotective effect of $\Delta^9$-THC was partially blocked by SR141716A, an antagonist/inverse agonist of CB1Rs, suggesting that the neuroprotective effects of these compounds are mediated significantly but not exclusively by this receptor. The possible mechanism(s) described for cannabinoid action involves activation of CB1Rs that are expressed in the inner neurons of the retina (ie. bipolar, amacrine and horizontal cells) (Yazulla et al., 1999). This leads to a consequent reduction of excitotoxicity by inhibiting voltage-sensitive $\text{Ca}^{2+}$ channels and/or by enhancing voltage-dependent $\text{K}^{+}$ channels activity (Pan et al., 1996; Twitchell et al., 1997), leading to decreased in $\text{Ca}^{2+}$ influx and membrane hyperpolarization. HU-211, a non-psychotropic cannabinoid agonist was also found to be neuroprotective after retinal ganglion optic nerve axotomy (Yoles et al., 1996). In addition, $\Delta^9$-THC and HU-211 have been shown to have anti-oxidant properties that are independent of CB1Rs and may provide neuroprotection by acting as scavengers for toxic reactive oxygen species produced following excitotoxic insult (Hampson et al., 1998; Marsicano et al., 2002).
1.6 Drug Delivery Systems

1.6.1 Drug Delivery Systems for Ocular Compounds

The most common form of ocular drug delivery is topical delivery into the cul-de-
sac. Optimal concentration of a drug at the site of its action depends on the appropriate
duration of contact of the drug with the cornea and is influenced by both the physical
properties of the drug as well as the physiological barriers.

Physiological barriers to the diffusion and absorption of a drug from the corneal
surface limit the entry of a drug into the eye. The cornea is composed of five layers, with
the epithelium and stroma being the major barriers for drug entry. More specifically, the
lipophilic cornea limits the entry of highly hydrophilic drugs, while the stroma is the rate-
limiting barrier for highly lipophilic compounds. In addition, the loss of solution to tear
fluid and lacrimal drainage, followed by absorption of the drug through the nasal mucosa
or the gastrointestinal tract into the systemic circulation limits the drugs bioavailability
(Lee and Robinson, 1986). Irritation of the eye by either the drug or vehicle due to
factors such as low pH, or hyper or hypotonicity often induces tearing and blinking which
further limits the amount of drug reaching ocular tissues (Järvinen et al., 2002; Sieg and
Robinson, 1977; Conrad et al., 1978). It has been reported that less than 5% of drug
solutions reach the anterior chamber of the eye (Kaufman, 1984; Järvinen et al., 2002).
Limited drug bioavailability to ocular tissues requires frequent dosing at very high
concentrations. This in turn results in higher absorption of drugs in the systemic
circulation and thereby increases the risks of side effects.
1.6.2 Delivery Systems for Cannabinoids

The best-known method of cannabinoid administration, with respect to drug bioavailability, is smoking. The high lipid solubility of the cannabinoid allows it to penetrate the alveolar membranes rapidly and avoids hepatic first pass metabolism. Peak plasma concentrations occur within the first 10 minutes following inhalation (Huestis et al., 1992; Chiang and Barnett, 1984; Lindgren et al., 1981; Ohlsson et al., 1980). However, the bioavailability of Δ9-THC is unpredictable and varies between subjects depending on the depth of inhalation, puff duration, breath-hold, and other factors. Furthermore, this route of administration is associated with significant exposure to mutagens, carcinogens and other products of pyrolysis (Matthias et al., 1997).

Oral administration of Δ9-THC (dronabinol) is another form of drug delivery, and has been available for clinical use for a number of years. However, this route for THC administration results in slow and erratic absorption, with peak plasma concentrations reported between 1 and 6 hrs (Perlin et al., 1985; Ohlsson et al., 1980). Furthermore, the bioavailability of oral Δ9-THC is poor (3-6%) due to extensive first-pass liver metabolism by cytochrome P450 enzymes.

Topical Δ9-THC and cannabinoid delivery to the eye appears to be the most convenient form of administration, and should result in rapid onset of action while minimizing possible adverse systemic side effects. However, the low aqueous solubility of cannabinoid compounds provides limitations for this route of administration. For example, cannabinoids are highly lipophilic substances with an octanol: water partition coefficient of 6000:1 (Gill, 1972). This lipophilicity makes intraocular penetration of the corneal epithelium and stroma difficult. Mineral oils, cyclodextrins, and microemulsion
have all been tested in topical cannabinoid research (Jarho et al., 1998; Jay and Green, 1983; Muchtar et al., 1992; for review see Järvinen et al., 2002). However, tissue irritation and limited and unpredicted bioavailability have been reported (Jay and Green, 1983; Green, 1998; Green and Kearse, 2000). Thus, there is further need to continue to develop and test alternative drug delivery systems for Δ9-THC and other novel cannabinoid drugs which are currently under development in order to provide an improved drug profile for the treatment of a variety of chronic pathologies, including glaucoma.

One system for Δ9-THC delivery that may provide to be useful in therapeutic applications is liposomes. Liposomes were first ‘discovered’ by Alec Bangham of the Agricultural Research Council’s Institute of Animal Physiology in Cambridge, England (1968) who noticed that phospholipids in an aqueous system form closed bilayered structures. Since then, liposomes have been used as drug carriers in animal and human studies for over 40 years.

Liposomes are microscopic vesicles composed of alternating aqueous compartments and phospholipid bilayers that can trap both hydrophilic and lipophilic drugs (Bangham et al., 1965). Hydrophobic/lipophilic drugs intercalate into the lipid bilayer(s) while hydrophilic drugs remain in the aqueous phase. Liposomes can be classified into four groups, depending on their size and the number of lipid bilayers: (1) large multilamellar vesicles (LMLVs), (2) small multilamellar vesicles (SMLVs), (3) small unilamellar vesicles (SULVs) and (4) large unilamellar vesicles (LULVs) (Figure 1.7). Liposomes resemble cell membranes in structure and composition. They are made
Figure 1.7  Physical structure of liposomes (A) Unilamellar vesicles are composed from single lipid bilayer surrounding an aqueous interior (B). Multilamellar vesicles are composed from two or more lipid bilayers, which are separated by aqueous phases.
from natural and biodegradable lipid molecules, including dipalmitoyl-phosphatidyl-
choline (DPPC), dipalmitoyl-phosphatidylglycerol (DPPG) and cholesterol and, therefore,
are virtually non-toxic (Myers et al., 1993; Thomas et al., 1991; Meisner et al., 1989).
Furthermore, liposomes can be modified to a particular size, composition and surface
charge in order to provide an optimum desired rate of drug delivery.

To date liposomal drug delivery has been limited in many applications by a short
circulation half-life and is affected by the route of administration. After intravenous
(i.v.) administration, liposomes are rapidly removed from circulation by mononuclear
phagocyte systems (MPS) including Kuffer cells of the liver and fixed macrophages of
the spleen (Allen et al., 1983). Some progress has been made to prolong the circulation
time of liposomes. For example, to achieve longer circulation time, the liposomal surface
has been coated with biocompatible polymers such as polyethylene glycol (PEG) that
slow down the recognition and clearance of liposomes (Kibano et al., 1990; Blume and
Cevc, 1993). In addition, gangliosides (GM1) or sphingomyelin have also been used to
increase circulation longevity of liposomes.

Liposomes can also be modified to increase drug accumulation in target tissues
and organs. For example, surface-attached ligands such as immunoglobulins (Ig) have
been included in liposomal preparations in order to allow for recognition and binding of
liposomes to cells of interest. Antibody-mediated liposome targeting of specific tissues,
such as tumour cells, provides another form of liposomal drug delivery with considerable
success.
1.6.3 Clinical Application of Liposomes

Liposomes have been used as drug carriers in the clinical setting for a number of years with drugs such as doxorubicin (Myocet, Sopherion) for the treatment of solid tumors in patients with breast metastases (Symon et al., 1999), vincristine (Onco TCS) for treatment of non-Hodgkin’s lymphoma and amphotericin B (AmBisome, Fujisawa Healthcare) for the treatment of systemic fungal infections (Lopez-Berestein et al., 1985; Sundar et al., 2003). Liposomes are also being explored as possible carriers for vaccines with Epaxal (Berna Biotech) – a Hepatitis A vaccine being available on the market. Liposomal preparations have been administered by IV, IM, pulmonary, oral and topical routes with enhanced bioavailability of the entrapped drug. The pulmonary delivery of liposome-encapsulated drugs seems to provide an especially promising route for drug delivery. Liposomal preparations delivered to the lungs are well tolerated (Myers, et al., 1993; Oyarzun and Baritussio, 1980) and result in a prolonged systemic absorption and high bioavailability of the administered drug (Taylor et al., 1989; Hung et al., 1997). In addition, pulmonary administration avoids hepatic first pass metabolism, again increasing the bioavailability of a drug.

With respect to ocular treatments, liposomes have been used to entrap antiviral and antimicrobial drugs to treat disorders of both the anterior and posterior segments of the eye (Schaeffer and Krohn, 1982). The liposomal manipulation for targeting the anterior segment of the eye has focused on achieving sufficient corneal penetration while the posterior segment applications concentrate on providing longer clearance time and reduced toxicity to ocular tissues (Frucht-Perry et al., 1992). The corneal absorption of
liposomal preparations and increased bioavailability has been shown to be superior for positively charged and large liposomes (Grass and Robinson, 1988; Guo et al., 1980). In addition, ganglioside-containing liposomes or liposomes with increased cholesterol content increase the penetration of drugs through the cornea (Schmidt-Erfurth et al., 1996; Barber and Shek, 1986). *In vivo* studies of liposomal antibiotic and antifungal preparations, such as penicillin, gentamicin and amphotericin B targeted into posterior segments of the eye show slower clearance and reduced toxicity upon intravitreal administration (Fiscella et al., 1987; Fishman et al., 1986; Rao et al., 1989).

In recent years a liposome-encapsulated Δ9-THC (LTHC) formulation has been developed (Hung et al., 1999; Hung et al., 1995). The incorporation of Δ9-THC into liposomes has a number of advantages over the existing delivery systems such as oral administration or smoking of the *Cannabis* plant. It allows for the delivery of a single drug of defined purity at a specified concentrations thus avoiding exposure to other components of the plant, especially those that are carcinogenic. In addition, LTHC prolongs the inactivation of the drug by enzymatic activity. The liposomal "environment" enhances the delivery of hydrophobic THC through tissue barriers, providing higher bioavailability for target organs, and at the same time reduces the side effects/toxicity seen after administration of free drugs.
1.7 Rationale and Specific Objectives

A growing body of evidence suggests an important role for cannabinoid systems in different physiological processes. Yet, scientific research into the clinical efficacy of cannabinoid compounds has been hampered by the lack of a suitable delivery system and the psychotropic side effects.

The hypothesis tested in this thesis are that the liposomal administration (i.t., i.v., and topical) of Δ9-THC will provide therapeutic levels of the drug in rat/rabbit ocular tissues and plasma, and will result in a decrease in IOP by mechanisms that is partially mediated through CB1Rs, which are expressed in the outflow tissues such as the TM.

The overall aim of this thesis was to evaluate the effectiveness of liposomal encapsulation of Δ9-THC as a potential drug delivery system for cannabinoids with respect to its pharmacodynamic and pharmacokinetic profiles.

The specific experimental objectives were: (1) To determine the pharmacodynamic profile for LTHC by examining the effect of LTHC on IOP, in Brown Norway rats before and after i.t. and i.p. administration of the drug and comparing this to the action of other cannabinoid agonists and pharmaceutical agents, commonly used in the treatment of glaucoma; (2) To investigate the bioavailability of Δ9-THC in ocular tissues and the plasma concentration (C_{THC}) of the compound versus time, after i.t., i.v. and topical delivery in the New Zealand white rabbit eye; and (3) To explore a possible cellular mechanism involved in the control of IOP by cannabinoid compounds in human TM cells, which line the outflow pathway for AH.
CHAPTER 2

Methods
2.1 Pharmacokinetics and Pharmacodynamics of *In Vivo* Administration of Liposomal Delta 9-Tetrahydrocannabinol

2.1.1 Animal Subjects

Brown Norway rats (experimentally naïve, 300g, males) and New Zealand White rabbits (2.5-3.0 kg, males) used in the experiments were obtained from Charles River (Montreal, Canada). Rats were housed 2 per group, while rabbits were housed in a group of 4-12, freely moving within the enclosed area. Both species were maintained on a 12 hours (hrs) light/dark cycle (lights off at 19:00 hr) and had *ad libitum* access to rat or rabbit chow and water. Ethical approval for this study was obtained from the Dalhousie University Committee on Laboratory Animals. All animals were treated accordingly to the standards and procedures set forth by the Canadian Council on Animal Care (www.ccac.ca).

2.1.2 Pharmacological Agents Used

Liposomal delta 9-tetrahydrocannabinol (Δ9-THC) (LTHC) was prepared and supplied by Defence Research and Development Canada (DRDC) (Department of Operational Medicine, DRDC, Toronto, Ontario).

WIN55,212-2 (Tocris, MO, USA) a synthetic cannabinoid was dissolved in a vehicle solution of 45% 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) (Research Biochemicals, Natic, MA). SR141716A (Sanofi-Synthelabo Recherche (Montpellier, France), a selective cannabinoid 1 receptor (CB1R) antagonist was dissolved in 45% 2-
HP-β-CD. R-(-)-MA (RBI, MA, USA) an analog of the endogenous cannabinoid, anandamide, was dissolved in 45% HP-β-CD.

Commercial preparations of 0.5% timolol solution (5 mg/ml) (Sabex, Boucherville, QC, Canada) and 0.005% latanoprost (Pharmacia & Upjohn, Kalamazoo, MI, USA) were obtained for topical (ocular) administration.

2.1.3 Liposomal-Encapsulated Δ9-THC and Control Liposomes

LTHC used in these experiments was prepared by Defence Research and Development Canada (DRDC) (Department of Operational Medicine, DRDC, Toronto, Ontario) according to previously published methods (Meisner et al., 1989; Hung et al., 1995; Hung et al., 1999), and was composed of dipalmityl-phosphatidylcholine plus cholesterol (Avanti Polar Lipids, Alabaster, AL) in a 7:3 molar ratio. In formulating the LTHC, the phospholipids were dissolved in a minimal volume of chloroform in a round-bottomed glass vessel, followed by the addition of a defined amount of cannabinoids (Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada). Chloroform was then evaporated under a stream of helium at 40°C and the glass vessel was placed under vacuum for 12 hrs to remove any residual solvent. The dried lipid-cannabinoid mixture was then hydrated at 51°C in phosphate-buffered saline (PBS) (0.15 M, pH 7.2) and kept at this temperature with periodic vortexing for 30 minutes to form unilamellar vesicles. The liposomes with entrapped cannabinoid were extruded 10 times with a thermobarrel extruder (Lipex Biomolecules, Vancouver, BC) fitted with doubly stacked polycarbonate filters of 400 nm pore size, using a helium pressure of 100-200 lb/in². The extrusion process produces a preparation with a more homogeneous liposome size distribution.
Liposome vesicle size was determined with the use of a Coulter N4SD particle-size analyzer. The preparation contained 0.6 mg of Δ9-THC per ml with 100 mg of lipid per ml. The percent drug encapsulation of LTHC was greater than 90%. For liposome controls, vesicles were prepared as described above except without the inclusion of Δ9-THC.

2.1.4 Routes of Drug Delivery

Routes of drug delivery used in the in vivo experiments included: intratracheal (i.t.) administration (used in both rat and rabbit experiments), intraperitoneal (i.p.) administration (used in rat experiments), intravenous (i.v.) administration (used in rabbit experiments) and topical (ocular) administration (used in rabbit experiments).

Procedures for i.t. delivery of LTHC in rats and rabbits were as follows. Immediately prior to the administration of LTHC a baseline (time zero) IOP measurement (described below, section 2.1.5) was recorded for each animal. Animals were then anesthetised with halothane (Halocarbon Laboratories, Diver Edge, NJ, USA), administered by inhalation delivered through an appropriately sized manifold (nose-only exposure), at a dose of 1.5 l.min⁻¹ O₂/4.5% halothane for induction followed by a dose of 1.5 l.min⁻¹ O₂/2.5% halothane for anesthesia maintenance. Once deeply anesthetized (determined by a loss of foot reflex), the trachea of animals was intubated under vision using a laryngoscope (#1 Miller). Intubation was then immediately followed by i.t. instillation of the LTHC preparation through the endotracheal tube using a hydraulic microsprayer that generates an aerosol with a mass median diameter (MMD) of 18 μm at an operating pressure of 2000 psi (PennCentury™ Microsprayer; Philadelphia, PA,
USA). The microsprayer allows local pulmonary delivery of drug with close to 100% efficiency (Beck et al., 1999) and has been successfully employed in rodents with minimal respiratory distress (Rees and Brain, 1995; Rees et al., 1999; Van Helden et al., 1997). Following each drug administration animals were mechanically ventilated and the chest auscultated to ascertain even distribution of the drug. The endotracheal tube was then removed and the animals were allowed free access to food and water after recovery from the anesthesia. Animals were fully conscious and mobile within 5 minutes after intratracheal drug delivery. For rats, LTHC doses ranging 0.01-1.0 mg.kg⁻¹ or liposomal control were employed. In rabbits, a 150µg dose of LTHC (delivered in a total volume of 250 µl) or an identical dose of the liposome control was used.

For i.p. delivery in rats LTHC (or the control liposomes) was administered at doses ranging from 0.01-1.0 mg.kg⁻¹ using a 26 gauge needle. The liposomal vesicles served as a control. The animals remained fully conscious throughout all the experiments. The cannabinoid agonist WIN55,212-2 was administered i.p. to rats at doses ranging from 0.017, 0.17, 1.7 and 17 mg.kg⁻¹ (0.5 ml total volume) using a 26 gauge needle. The vesicle, HP-β-CD, was used for the control (0.5 ml total volume). R-(+)-MA, an analog of the endogenous cannabinoid AEA, was administered to rats i.p. at doses of either 1.7 or 2.5 mg.kg⁻¹. In all experiments an equal volume of vehicle was used as control. In experiments employing the cannabinoid antagonist SR141716A, a dose of 2.5 mg.kg⁻¹ (delivered in total volume of 0.5 ml) was administered via an i.p. route, 30 minutes prior to administration of LTHC, WIN55,212-2 or MA.

Topical ocular delivery of LTHC (30 or 60 µg doses; 50 or 100 µl, respectively) was employed in rabbits. During this procedure animals were restrained in a commercial
rabbit restrainer. The drug was instilled into the right eye of each rabbit. During application, the rabbit’s upper eyelid was pulled slightly from the eye to ensure that only a minimal amount of drug was lost due to blinking. The left eye was untreated and served as a control.

To compare the IOP-lowering effect of LTHC alone to that of LTHC combined with 0.5% timolol or 0.005% latanoprost, the study drugs were administered at the same time unilateral to the right eye of each animal, while 0.2 mg.kg⁻¹ of LTHC was administered by IP route. The IOP measurements were taken as described below.

An intravenous (i.v) route of LTHC delivery was also used in rabbits. In this procedure animals were first placed under general anesthesia (as described above for intubation) and 150 µg (in a total volume of 250 µl) of LTHC was injected using a 26-guage needle into the right marginal ear vein of each rabbit. After drug administration animals were allowed to fully recover.

2.1.5 Measurement of Intraocular Pressure in Rats and Rabbits

Intraocular pressure (IOP) was measured in rabbits and rats using a hand-held electronic tonometer (Tono-Pen®XL, Mentor, Norwell, MA). The tonometer works on the principle that the force required to flatten a given area of the cornea is proportional to the pressure inside the eye. Previous experiments using Brown Norway rats have shown excellent correlation between IOP measurements made with Tono-Pen®XL and the more invasive transducer method of IOP determination (Pan et al., 2000). Prior to IOP measurement rats were placed on a stable surface and restrained lightly by hand, rabbits were restrained in the commercial rabbit restrainer (Techniplast, Italy). Then, 0.05%
tetracaine eye drops (Dioptic Laboratories, Markham, Ontario) were applied topically in order to produce local anesthesia. During IOP measurements the tonometer was held perpendicular and placed gently against the anesthetized surface of the cornea. For each timed measurement (described below) IOP was measured ten times and a mean value was determined and reported. Animals were handled and exposed to the IOP measurements at least five days prior to the experiment.

In a typical experiment a baseline IOP (time 0) (Figure 2.1) was determined (baseline IOPs ranged between 18-22 mmHg for rats and rabbits). Immediately following this measurement, drugs were administered as described above (section 2.1.4). Subsequent IOP measurements in rats were taken at 15, 30, 45, 60, 75, 90, 105 and 120 minutes after drug administration. IOP measurements in rabbits were taken at 15, 30, 60, 120 and 240 minutes post drug administration. All experiments were carried out at precisely the same time in the morning in order to ensure that any changes in IOP readings were not a consequence of diurnal changes in IOP readings reported to occur naturally (refer to the Figure 2.1).

The Analysis of the IOP Measurements

These continuous data were analyzed using One Way ANOVA analysis with post-hoc multiple comparison using Tukey test. $P<0.05$ was considered statistically significant. The data presented in all the figures represent mean ± standard error of the mean (SEM). $ED_{50}$, the dose of agonist producing 50% maximal change in IOP ($\Delta$IOP), was calculated by fitting dose-response data for the maximum $\Delta$IOP with a variable Hill slope given by parameter “p” where
Figure 2.1. Typical protocol for IOP measurements used in Brown Norway rats and New Zealand White Rabbits.
Anesthetic drops instilled in the eye

↓

Baseline IOP measurement with tonopen

(time 0, baseline)

↓

Delivery of drug

(e.g. WIN55,212-2, MA, LTHC)

↓

IOP measurements with Tonopen
y = A1 + (A2-A1)/1+10\(^{(\log X_0-x)p}\) \hspace{1cm} (Equation 1)

Where A1 = ΔIOP with liposomes or vehicle control, A2 = ΔIOP with maximal dose of agonist tested, LogX0 = 50% of A2 (ED\(_{50}\)) and p = hill slope.

2.1.6 LTHC Pharmacokinetics in Brown Norway Rats and New Zealand White Rabbits

**Blood Sampling**

Pulmonary absorption and disposition following i.t. delivery of LTHC were assessed in rat blood. Animals were anesthetized with halothane (Halocarbon Laboratories, Diver Edge, NJ, USA) and 0.2 mg.kg\(^{-1}\) of LTHC was administered i.t. (as described above, section 2.1.4). While under anesthesia 3 ml of blood was obtained from each animal using a cardiac puncture at time 0 (immediately prior to drug delivery (baseline), or at 15, 30, 45, 60 or 120 minutes post-LTHC delivery. Immediately following final blood collection the rats were sacrificed by cervical dislocation. The collected blood was heparinized (Leo Pharma Inc., Thornhill, Ontario) and the plasma was immediately separated and stored at -20°C until analyzed. Plasma Δ9-THC concentrations were determined using a modified gas-chromatography/mass spectrometry (GC/MS) technique, as described by Frederick and Fowler (1985). All Δ9-THC assays were carried out by DRDC (Toronto, Ontario, Canada). The pharmacokinetic profiles for LTHC were examined for the three routes of LTHC administration that were used in rabbits. All rabbits, regardless of the route of LTHC delivery used, were restrained in a commercial rabbit restrainer (Techniplast, Italy) and topical ocular tetracaine (0.05%) was applied. At this time IOP was determined using the Tonopen. Animals were next
placed under general anesthesia via intramuscular (i.m.) injection of 30 mg.kg\(^{-1}\) ketamine and maintained under anesthesia with halothane (2.5-4.0 %). Once anesthetized, the central ear artery was cannulated using a 22-gauge IV catheter (Johnson & Johnson Medical\textsuperscript{TM}, Arlington, Texas, USA). A time 0 blood sample (baseline) was taken and LTHC was then administered. Within 5 minutes after drug administration the animals had fully recovered and IOP’s were taken at time points 15, 30, 60, 120 and 240 minutes after LTHC administration, this was followed by immediate blood sample collection. The blood was heparinized and the plasma was separated and stored at \(-20\) C\(^\circ\) until analyzed. With the exception of the 0 time point, the animals remained fully conscious during the blood collection.

In these pharmacokinetic experiments, LTHC was delivered i.t. (150 \(\mu\)g LTHC (250 \(\mu\)l volume) or control liposomes, as described above in section 2.1.4). For i.v. delivery, 150 \(\mu\)g (250 \(\mu\)l) LTHC was injected using a 26-gage needle into the marginal ear vein of each rabbit, immediately following anesthesia. For the topical ocular delivery of LTHC, 30 or 60 \(\mu\)g was dropped into the right eye of each rabbit (as described above, section 2.1.4). The left eye of each rabbit served as a contralateral control.

\textit{Tissue Sampling}

Following LTHC administration blood was collected and rabbits were sacrificed using an overdose of pentobarbital sodium (~2 ml) (Butler, Columbus, OH) at either of the following time points; 0, 15, 30, 60, 120 and 240 min. The eyes were enucleated and gently washed in phosphate buffer saline (PBS). The enucleated eyes were dissected on ice using sterile instruments and the eye tissues, including the ciliary body, retina, lens,
cornea and vitreous humor (VH), were harvested, placed in pre-weighed glass tubes, weighed and frozen at -20°C until analysis. The Δ9-THC levels in the various eye tissues were determined by GC/MS, carried out by DRDC (Toronto, Ontario, Canada) (see Figure 2.2).

2.1.7 Pupil Diameter Measurements

New Zealand White rabbits were restrained in the commercial rabbit restrainer (Techniplast, Italy) and the pupil diameter of each rabbit was measured with a millimeter ruler under standard laboratory light. Then, 60 µg (100 µl volume) LTHC (n=3), or liposomal control (n=3), was applied unilaterally to the right eye of each animal. The measurements of pupil diameter were taken every 15 minutes over a period of 240 min, following drug administration. The data was analyzed using paired t-test.
Figure 2.2. Typical protocol used to determine the pharmacokinetic profile for LTHC in New Zealand White rabbits.
Administration of LTHC
(Topical, i.t. and i.v.)

↓

Animals Sacrificed

↓

Eye Tissues Harvested
(Ciliary Body, Retina, Lens, Cornea, Vitreous Humor)

↓

Gas Chromatography/Mass Spectrometry
2.2 Cellular Mechanisms of Cannabinoids in Ocular Tissues

2.2.1 Cell Lines and Culture Techniques

Both rabbit non-pigmented ciliary epithelial (NPCE) and human trabecular meshwork cell (HTM5) lines were used in a set of experiments in order to determine the expression of various cannabinoid receptors and also to identify possible mechanisms which are involved in the action of cannabinoid compounds.

The NPCE cells were derived from simian virus-40 (SV40) - transformed rabbit NPCE. Briefly, to develop immortalized NPCE lines, the ciliary body epithelium (CBE) tissue was transformed with SV-40 virus, generating transformed cells that are capable of proliferation for many passages (Coca-Prados and Wax, 1986). Positive viral transformation of primary cultured rabbit NPCE cells was verified by expression of the large T-antigen (T-Ag) in the nuclei of SV40-transformed cells. The SV-transformed NPCE cells maintain functional β-AR (Coca-Prados and Wax, 1986), as well as the functional ion transporters, such as K channels and Cl channels (Helbig et al., 1989c).

NPCE cells were maintained at 37°C in standard culture media that included Dulbecco’s Modified Eagle’s Medium (DMEM) (Canadian Life Technologies, Burlington, ON), 10% fetal bovine serum (FBS) and 1% gentamicin, in an atmosphere of 5% CO2, 95% O2. The culture media was changed every 3-5 days and cells were passaged after reaching > 80% confluence.

The human trabecular meshwork (HTM5) cell line was derived from primary human trabecular meshwork cells, which were transformed by transfection with an origin defective mutant of SV40 virus (a kind gift of Dr. Pang of Alcon Laboratories, FT. Worth, TX, USA). TM5 cells retain the properties of primary TM cells (Pang et al.,
HTM5 cells were maintained in tissue culture flasks (750 cm²) (Invitrogen, Burlington, ON) in standard culture media containing DMEM supplemented with 10% fetal bovine serum (FBS) and 1 mg/100 ml gentamicin (Sigma Chemical Company, St. Louis, MO, USA). The cells were maintained in a 37°C incubator in an atmosphere of 5% CO₂ and 95% O₂. The culture media was changed every 3-5 days and cells were split when they reached confluency.

2.2.2 Isolation of Total RNA from Cultured Cells and Tissue

Total RNA was isolated from NPCE cells, HTM5 cells, the ciliary body epithelium and the retina of both rats and rabbits.

For cultured cells, NPCE or HTM5 cells were plated onto 3.5 cm diameter dishes at a density of 10⁵ cells/ml and were allowed to grow until they were ~ 80% confluent. Total RNA was isolated from either NPCE or HTM5 cells using Trizol Reagent (Life Technologies, Burlington, ON) according to the manufacturer’s protocol and as previously described by Shi et. al. 2003. Ocular tissues, including the isolated ciliary body epithelium and retina, were homogenized in 200 µl Trizol in a 1.5 ml Eppendorf tube with a tissue homogenizer (maintaining temperature at 4°C throughout all procedures). An additional 800 µl of Trizol was added to each sample and the samples were vortexed.

Cell or tissue homogenates were incubated for 5 minutes at RT in order to dissociate the nucleoprotein complex. In order to allow the RNA to dissolve in aqueous phase chloroform (200 µl) was added to the samples, which were then incubated for a further 2-3 min. The samples were centrifuged at 4°C for 15 minutes at 12,000 rpm and
the upper aqueous phase was removed and transferred to clean Eppendorf tubes. Samples were mixed with 500μl isopropyl alcohol and incubated for 10 minutes at RT to precipitate the RNA. Samples were then centrifuged at 4℃ for 15 minutes at 12,000 rpm and the supernatants were discarded. The RNA pellets were washed twice with 75% ethanol, the pellets were air dried and dissolved in 16 μl of RNase-free water. The concentration and purity of RNA was measured using a spectrophotometer, with absorption of the samples at A_{260}/A_{280} nm (RNA vs protein).

2.2.3 Reverse Transcriptase-Polymerase Chain Reaction

The RT-PCR (reverse-transcription-polymerase chain reaction) technique was used to determine mRNA expression of CB1R in rat ciliary body epithelium and retina, as well as in the cultured HTM5 cell line.

The reverse transcriptase reaction generates a single stranded DNA molecule complimentary to the RNA (cDNA) from ocular tissues. To rule out potential DNA contamination, the same RT reaction was carried out in the absence of RT, the product of which was also used as a template for subsequent PCR reactions.

The cDNA was then used as a template in PCR reactions with primers specific for CB1R or cyclophilin. Cyclophilin is a constitutive housekeeping gene used as an internal control for the PCR reactions. The primers for amplification of the human CB1R were: 5' - TGCAGGCTTTACCACTTCATC-3' (forward, bp 536-559) and 5' - GACGTGTGGATGATGCTCTTC-3' (reverse, bp 1056-1033), GenBank#:XM 004350). PCR conditions for amplification of the CB1R included: denaturation at 94℃ for 1 min, 35 cycles of denaturation at 94℃ for 1min, annealing at 56℃ for 1minutes
and extension at 72°C for 1 min, followed by final extension at 72°C for 7 min. RT-PCR amplification of cyclophilin was also performed as an internal control and was used to normalize CB1R mRNA expression. Primers for cyclophilin amplification were designed from a segment of cyclophylin cDNA from 44 to 414 bp (forward: 5'-TGG TCA ACC CCA CCG TGT TCT T- 3'; reverse 5'-GCC ATC CAG CCA CTC AGT CTT G-3'). The PCR conditions for amplification of the cyclophylin protein included: denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min.

The PCR product and DNA ladders were separated using an agarose E gel in Tris-Borate EDTA buffer (TBE buffer) (Sigma-Aldrich Co., Baltimore, MD, USA). The PCR product gel was visualized under ultraviolet illumination. Positive PCR bands of the excepted size were excised from the gel, and purified using QIAquick Gel Extraction Kit (QIAGEN Inc., Mississauga, Ontario, Canada)

2.2.4 PCR Cloning and Transformation

The cloning of PCR products was performed using the pGEM®-T vector system I (Promega, Madison, WI, USA). The pGEM-T is a linear plasmid, which contains a cloning site that has many restriction enzyme sites, which are used to insert the DNA of interest. The multiple cloning site is in the middle of a reporter gene lacZ, which encodes for the enzyme β-galactosidase that in turn degradates X-gal and allows for screening for the bacteria that has been transformed with insert. In addition, pGEM-T plasmid contains a selectable marker, a carbenicillin-resistant gene that allows the bacteria transformed
with the plasmid to be selected by growing the bacteria on a plate that contains carbenicillin.

The ligation was obtained by incubating the purified PCR products, pGEM-T vector and T4 DNA ligase in ligation buffer (LB) (Table 2.4) at 4°C overnight. The ligated DNA was then purified using an ethanol precipitation method, which removes MgCl₂ and polyethylene glycol (PEG). The purified CB1R DNA was then used to transform the bacterial cells through electroporation.

Transformation of bacterial cells with pGEM-T plasmid was carried out using electroporation. Specifically, 3 μl of purified pGEM-T vector and 40 μl of HO1 *E.coli* bacterial cells were placed into a chilled cuvette and mixed by pipetting up and down. The samples were then placed into a microelectroporation chamber. An electrical pulse was applied to charge the chamber. The electrocompetent cells were transformed with ligated pGEM-T vector at 2.5 kV with a capacitance of 25 μF and a resistance of 125 Ω. The successful time course was between 4.5-4.6 sec. Electroporated bacterial cells were then recovered and grown by incubating in LB media for 1 hr at 37°C on a shaker, at a speed of 250 rpm. The bacterial cells were then spread onto LB agar plates containing X-gal and antibiotic. The plates were sealed with a strip of parafilm and were incubated at 37°C overnight.

The bacterial colonies of *E. coli* were then screened for appearance. Briefly, the colonies that were successfully transformed with the insert were not able to produce functional β-galactosidase enzyme (a product of the *lacZ* gene), and therefore did not degrade X-gal, so these appeared white. The bacterial colonies that contained the religated vector/insert “free” plasmid, and therefore functional β-galactosidase, produced
blue colonies. Furthermore, only those bacteria that have been transformed with pGEM-T successfully survived on the carbenicillin-positive plates.

The white colonies were then segregated and amplified by PCR reaction, in order to confirm the identity of the PCR product. In addition, white colonies were inoculated in 4 ml of LB medium containing antibiotic and allowed to grow at 37°C in a shaker at a speed of 250 rpm, overnight. Next, the LB medium and bacterial cells were divided in half: 2 ml of bacterial cells were centrifuged (5000 rpm), the resulting pellet was dissolved in 15% cold glycerol and was frozen at -80°C. The remaining 2 ml was used to purify the plasmid DNA using the GenElut™ Plasmid Miniprep Kit (Sigma-Aldrich Canada, Oakville, ON).

Restriction enzyme digest analysis was performed, to confirm the identity of the PCR product insert. The oligonucleotide sequence of the PCR product was determined using a T7 sequencing kit and M13 universal forward and reverse primers (Pharmacia, Amersham, Baie d'Urfe, QC).

2.2.5 Immunohistochemistry and Immunocytochemistry

For immunohistochemical tissue preparation animals were sacrificed with euthanol (~2 ml). In vivo removal of blood and fixation of eyes were carried out with sequential perfusion with ice cold PBS (pH 7.4), and 4% paraformaldehyde (PFA). Subsequently, the eyes were enucleated, post-fixed in 4% PFA for 24 hours at 4°C, cryoprotected in 30% sucrose solution (>48hrs) and immersed in gelatin at 37°C overnight. Eyes were then embedded in a block of gelatin, post-fixed in 4% PFA, and
placed in 30% sucrose, at 4°C. The eyes were sectioned at a thickness of 25-30 μm on a freezing microtome (Leica, SM 2000R).

For immunohistochemistry, coronal sections of rat eyes were gently washed in PBS. Then, sections were blocked with 10% normal goat serum and incubated with the CB1R primary antibody overnight at 4°C (1:500 dilution; Chemicon International, Temecula, CA, USA). The following day, the sections were washed in PBS (3x15 min) and incubated in a 1:1000 dilution of fluorescent conjugated goat anti-rabbit antibody (CY™3) (Chemicon; Temecula, CA, USA) for 1 hr at RT. After a series of washes with PBS (pH 7.4) the sections were mounted onto slides and were viewed with a confocal microscope.

Immunocytochemistry was carried on both the NPCE and HTM5 cell lines. Cells were gently washed with PBS and fixed in 100% Methanol for 5 minutes at -20°C. Following fixation, the cells were washed with PBS (3x15min) and the cell membranes were permeabilized in 0.3% TritonX-100 for 20 min, at RT, followed by 10% normal goat serum (Vector Laboratories Inc., Burlingame, CA, USA) for 1 hr to block non-specific staining. Primary antibodies were directed against either CB1R (1:500 dilution; Chemicon International, Temecula, CA, USA), dimerized CB1R (L14) (1:1000 dilution; a kind gift of Dr. Ken Mackie, University of Washington, Seattle, WA, USA), muscarinic receptor 3 (M3R) (1:500 dilution; Biodesign International, Saco, ME, USA), extracellular signal-regulated kinase 1/2 (ERK1/2) (1:500 dilution; Santa Cruz Biotechniolgy, Santa Cruz, CA, USA), α-Smooth Muscle Actin (α-SMA) (1:300 dilution; Sigma Aldrich, Oakville, Ontario, Canada) and vinculin (1:500 dilution; RDI, Concord, MA, USA). Primary antibodies were diluted to the desired concentration and the cells were incubated
overnight at 4°C, on a shaker platform. For negative controls, the primary antibody was omitted and cells were incubated in PBS. The next day the cells were washed (3x15min) in PBS, and incubated in fluorescent conjugated secondary antibody (CY\textsuperscript{TM}3; Chemicon, Temecula, CA, USA) for 1hr, at RT. Finally, the cells were washed in PBS (3x15min), mounted onto glass slides with aqueous mount (Sigma-Aldrich Co., Oakville, Ontario, Canada) and the staining was visualized with confocal microscopy.

For actin staining of the HTM5 cell line, a fluorescent conjugate of phalloidin was used in order to visualize and detect any changes in actin filaments organization after treatment with either WIN55,212-2, Carbachol or KCl. Phalloidin is a fungal toxin that binds only to polymeric and oligomeric forms of actin, thereby strongly stabilizing the actin. Stock solutions of Phalloidin-FITC P5282 (Sigma-Aldrich Co., Oakville, Ontario, Canada) were made in DMSO at 0.1 mg/100 μl. Final dilutions of 1-10 μM concentrations were made in PBS.

Cells were washed with PBS and then exposed to either WIN55,212-2 or KCl for 30 sec-10 minutes intervals. They were then gently washed at 4°C in PBS and fixed for 5 minutes in 4% PFA. The HTM5 cells were then washed (3 x 15 min) in PBS, incubated in 0.1% Triton X-100 for 20 minutes at RT and stained with fluorescent phalloidin-FITC for 1 hr at RT. After which they were washed 4 x 15 minutes with PBS to remove any unbound phalloidin. The staining was visualized using a confocal microscope.
2.2.6 Western Blot Analysis

Ciliary body epithelium tissue was dissected from rat and rabbit eyes and homogenized in lysis buffer (LB) (Table 2.4) containing protease inhibitor using a tissue homogenizer ("Caframo" stirrer R2R11-64; Wiarton, Ontarion, Canada) at 4°C. Protein analysis was performed using the Micro BCA™ Protein Assay Reagent Kit (Chromotographic Specialities, Brockville, Ontario). Protein samples (25-40μg) were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Tables 2.6 and 2.7) in running buffer (Table 2.3) for approximately 1 hour at 100V and transferred to Immobilon™-P PVDF membrane (Millipore Corporation, Bedford, MA, USA), in transfer buffer (Table 2.4), overnight at 35 V. The membranes were air dried at RT for 2 hrs, and treated with PBS blotto (Table 2.9) containing 5% bovine serum albumin (BSA) for 1 hr at RT. Polyclonal antibodies for CB1R (1:500 dilution; Chemicon International, Temecula, CA, USA) were diluted in the same solution and the membranes were incubated at 4°C overnight on a rocking platform. After repeated washes with PBS-Tween 80 buffer (containing 2% Tween 80), the membrane was incubated with peroxidase-conjugated secondary antibody (1:1000 dilution) for 1 hr at 37°C on a rotator. After three final washing steps (15 min, PBS-Tween 80) the membranes were washed with PBS-Tween 80 buffer for 3 x 15 min. The immunoreactive proteins were then visualized by chemiluminescence using the ECL plus system (Amersham Life Science, Little Chalfont, Buckinghamshire, England), according to the manufacturer's instructions, and were recorded on high performance chemiluminescence film (Amersham Bioscience, Little Chalfont, UK).
For cultured cells, confluent monolayers of HTM5 cells were treated with either cannabinoid ligands or other specific drugs known to affect the contractility and relaxation of these cells, for 5 or 15 minutes at 37°C. Cells were then washed in ice cold PBS, scraped, transferred into Eppendorf tubes, centrifuged at 5000 rpm, at 4°C, for 1 minute and resuspended for 20 minutes in 100 μl of LB, containing 40 μl/ml of protease inhibitor. Subsequently, the samples were centrifuged at 4°C, for 15 minutes at 13000 rpm, the supernatants were collected and the protein content was determined using the BCA™ Protein Assay Kit (Biolynx Inc., Brockville, Ontario). Equal amounts of proteins (40 μg), and molecular markers were subsequently resolved using a 12% SDS-PAGE gel electrophoresis (Tables 2.6 and 2.7), for 1 hr at 100 V. Next, the proteins were electro-transferred to Immobilon™-P PVDF membrane (Millipore Corporation, Bedford, MA, USA) overnight at 35 V, in transfer buffer (Table 2.4). Immunodetection was carried out using polyclonal antibodies for myosin light chain (MLC) (1:500 dilution; Abcam Inc., Cambridge, MA, USA) and pERK (Tyr204)-R (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in PBS, containing 5% skim milk, followed by 3 x 15 minutes washes in PBS, and subsequent exposure of membranes to peroxidase-conjugated secondary antibodies for 1 hr, at room temperature. The signals were visualized using a chemiluminescence kit (ECL™ Western Blotting Detection reagents, Amersham Bioscience, England) digitalized into Kodak ID Scientific Imagining System (Eastman Kodak Company, New Havens, CT, USA) and analysed with the UN-SCAN-IT for Windows (Silk Scientist, Orem, Utah, USA). Statistical analysis was performed by using the unpaired T-test.
Table 2.1.  Composition of Sample Buffer (100 ml)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>10 ml</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>30 ml</td>
</tr>
<tr>
<td>Upper-Tris (4x)</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>42.5 ml</td>
</tr>
</tbody>
</table>

Table 2.2.  Composition of Upper Tris (4x) (100 ml)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Weight or Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>6.06 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>4 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>96 ml</td>
</tr>
</tbody>
</table>

pH value adjusted to 6.8 with 12M HCl

Table 2.3.  Composition of Running Buffer (5x) (4000 ml)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Weight or Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>58 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>288 g</td>
</tr>
<tr>
<td>SDS</td>
<td>20 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>4000 ml</td>
</tr>
</tbody>
</table>

Diluted to 1x in distilled H₂O for working solution
Table 2.4. Composition of Lysis Buffer

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100</td>
<td>10 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>5 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.77 g</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>2.42 g</td>
</tr>
<tr>
<td>Deoxycholic Acid</td>
<td>5 g</td>
</tr>
</tbody>
</table>

pH value adjusted to 8.0. Total volume brought to 1 L.

Table 2.5. Composition of Transfer Buffer (pH 8.3-8.6) (4000 ml)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Weight or Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>12.12 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>57.6 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>800 ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>3200 ml</td>
</tr>
</tbody>
</table>

Table 2.6. Composition of TBS (3000 ml)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na Cl</td>
<td>24 g</td>
</tr>
<tr>
<td>Tris Base</td>
<td>7.26 g</td>
</tr>
</tbody>
</table>
### Table 2.7. Composition of 12% Lower Gel

<table>
<thead>
<tr>
<th>Compound</th>
<th>Weight or Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>3.18 ml</td>
</tr>
<tr>
<td>Lower Tris</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>4.18 ml</td>
</tr>
<tr>
<td>AP</td>
<td>150 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

### Table 2.8. Composition of Upper Stacking Gel

<table>
<thead>
<tr>
<th>Compound</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>3.17 ml</td>
</tr>
<tr>
<td>Upper Tris</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>500 µl</td>
</tr>
<tr>
<td>AP</td>
<td>80 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

### Table 2.9. Composition of Blotto (25 ml)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skimmed Milk</td>
<td>1.25 g</td>
</tr>
<tr>
<td>BSA</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Antifoam A</td>
<td>8.25 µl</td>
</tr>
<tr>
<td>Thimersol</td>
<td>A grain</td>
</tr>
<tr>
<td>PBS</td>
<td>25 ml</td>
</tr>
<tr>
<td>Compound</td>
<td>Weight</td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>NaCO3</td>
<td>1.59 g</td>
</tr>
<tr>
<td>NaHCO3</td>
<td>2.93 g</td>
</tr>
</tbody>
</table>
2.2.7 Optical Magnetic Twisting Cytometry

To evaluate changes in cell stiffness, which underlies the structural reorganization of actin filaments, Optical Magnetic Twisting Cytometry (OMTC) was used. The OMTC technique was developed in the laboratory of Dr. Geoffrey Maksym (School of Biomedical Engineering, Dalhousie University, Halifax, N.S., Canada) and the details of this technique have been published previously (Fabry et al., 2001; Smith et al., 2003). OMTC measures the changes in the cytoskeletal stiffness of cells by optically detecting the motion of individual magnetized beads in response to an externally applied force while the beads are connected to the cell cytoskeleton (CSK) through ligand-receptor linkages (described below). OMTC also allows detection of changes in cell stiffness following pharmacological stimulation with different pharmacological agents, which modify CSK organization.

The cell stiffness, \( G \) (measured in units of Pascal per nanometer) is defined from a ratio of a specific torque, \( T \), to the resultant bead displacement \( D \).

\[
G = \frac{T}{D} = G' + iG''
\]

Equation 2

Where \( G' \) is the in-phase component or elastic stiffness (referred to as cell stiffness, in Pascal/nm), \( G'' \) is the out of phase component (10-20% of the \( G' \)), and \( i \) is the unit imaginary number \( \sqrt{-1} \).

To measure cell stiffness in HTM5 cells, the ferromagnetic beads (4.5 \( \mu \)m in diameter) (a kind gift of Dr. Fredberg; Harvard School of Public Health) were coated
with synthetic RGD (Arg-Gly-Asp) containing peptide (Peptide 2000, Integra Life Sciences, San Diego, CA, USA) at a concentration of 50 μg per mg of beads in 1 ml of carbonate buffer (pH 9.4) (Table 2.10). The coated beads were centrifuged for 3 minutes at 1200 rpm, and the buffer was discarded. Then the beads were gently washed 3 times in PBS, and dispersed in serum-free DMEM, vortexed and warmed to 37°C. In order to determine the amount of beads to be added to each well containing cells, the first well was screened for appearance; the optimal number of beads per cell was 2-3 beads. Beads were then added to the remaining wells and the cells plus beads were incubated for 20 minutes at 37°C. The RGD-coating of the beads allowed them to bind to the transmembrane integrin receptors and form focal adhesions with the cells. In order to remove unbound beads, the cells were washed twice in DMEM, followed by addition of 100 μl DMEM media. Each well, containing the HTM5 cells with attached beads, was then placed in the twisting device composed of magnetizing and twisting coils fitted onto the stage of an inverted microscope (DM-IRB, Leica Microsystems, Germany) (Figure 2.3) and observed using a 20X objective (NA 0.40) and CCD camera (Cooke SensiCam). The beads were magnetized using large amplitude but short duration electric pulse (approx. 2 kV, 250 μs) (HIM-3000 Magnetizer) through a pair of Helmholtz coils which provided a magnetic field greater than 0.1 T. The cell-bead preparation was then exposed to the mechanical stimulation of 56 Pa specific torque at 0.3 Hz, with amplitude and frequency set at 1. Stiffness was measured for 30 sec before (control) and 30 or 90 sec after the administration of various pharmacological agents, or control (DMEM only). While the beads were twisted, the camera imaged the beads continuously (~200 beads at a time). The displacement of beads in response to the applied T was computed from the
recorded bead positions, after being digitally processed in order to eliminate any drift or noise of the beads due to possible erratic or irreproducible motions (see Fabry et al., 2001; Smith 2003). Bead positions on the image were determined using an intensity centroid algorithm. The resolution of the OMTC was 2.5 nm (rms) at 20X magnification.

Analysis of the OMTC Measurements

For each treatment group, 2-3 experiments were conducted, with 4-12 wells per treatment group. Approximately 200 beads were measured in each well. The stiffness is represented as the mean ± SEM, where the mean is the stiffness averaged over all wells in the group and SEM is the standard error of mean. To test for significant differences in cell stiffness between control and each experimental group, a paired t-test with 95% confidence level was used (p< 0.05).
Figure 2.3  (A) Simplified diagram of Optical Magnetic Twisting Cytometry (OMTC). OMTC is a small apparatus that consists of twisting and magnetizing coils, fitted on a microscope stage. The cultured cells with attached beads are placed inside of this apparatus. The OMTC optically tracts and records the bead motions while magnetically oscillating the beads, and therefore allows for the assessment of CSK stiffness. (B) Specific torque $T$, applied to bead results in bead displacement $D$; the ratio between these two defines the stiffness, $G$. 
CHAPTER 3

Results
3.1 LTHC Administration Reduces IOP in Rats and Rabbits and Provides Adequate Level of Δ9-THC in Ocular Tissues and Plasma

3.1.1 CB1R mRNA and Proteins are Present in Rat Ocular Tissues and Cells

To determine the presence of CB1R mRNA in rat ciliary processes and retina (positive control) RT-PCR amplification was used with primers specific for human CB1 receptor cDNA. A PCR product with a predicted size of 500 bp was detected in both the ciliary body tissue and retina (Figure 3.1A). The size of the PCR product was consistent with the expected size for CB1Rs using primers described in the methods section (see section 2.2.3).

Confirmation of the presence of CB1Rs in ocular tissue associated with aqueous humor production and outflow was obtained using immunohistochemical staining of a coronal section of rat eye using a polyclonal antibody directed against the human CB1R. The highest immunoreactivity for CB1Rs was detected in the ciliary body processes with strong labelling in the non-pigmented ciliary epithelial cells (Figure 3.1B). Moderate staining for CB1Rs was also evident in the radial muscles and trabecular meshwork. Control sections, with no exposure to primary antibody, showed no discernible staining in any of these structures (Figure 3.1C).

Figure 3.2A shows the expression of CB1R mRNA in the HTM5, NPCE cell lines and rat retina (positive control). As with ciliary processes tissues, a 498 bp PCR product was obtained with RT-PCR. Figure 3.2B&C shows Western blot analysis of the expression of monomeric CB1R of 53 kDa and a 250 kDa CB1R oligomeric complex, respectively. Immunohistochemical techniques using a polyclonal antibody directed against human CB1R confirmed the presence of CB1R protein in HTM5 cells (Figure
3.2D). The control, with no exposure to primary antibody, showed no staining in HTM5 cells (Figure 3.2E).

3.1.2 The Endocannabinoid Analogue, MA, Reduces IOP in Brown Norway Rats

Methanandamide, a more stable analogue of endogenous AEA, was used to examine whether endocannabinoid compounds affect IOP in Brown Norway rats. Figure 3.3 demonstrates that i.p. administration of MA produced an IOP-lowering effect in Brown Norway rats (n= 4-9). The peak reduction in IOP (mean ± SEM) with both 1.7 mg.kg⁻¹ and 2.5 mg.kg⁻¹ of MA was seen at 45 minutes after administration. Measurements ranged from 19.75 ± 0.386 to 17.925 ± 0.217 and from 20.183 ± 0.232 to 17.95 ± 0.157, respectively. The peak changes in IOP (ΔIOP, mean ± SEM) following 1.7 mg.kg⁻¹ and 2.5 mg.kg⁻¹ of MA were 1.825 ± 0.234 and 2.233 ± 0.212, respectively. The decrease in IOP by MA was blocked by pre-administration of the selective CB1R antagonist SR141716A. This suggests that cannabinoids act in the eye to produce a decrease in IOP that is mediated via CB1Rs.

3.1.3 LTHC Reduces IOP in Brown Norway Rats

The effect of LTHC on IOP after i.t and i.p. administration in Brown Norway rats was investigated to determine the pharmacodynamic profile of this liposomal cannabinoid preparation in lowering IOP. Figure 3.4A shows the change in IOP, measured in four to eight rats per group, after i.t. administration of different doses (0.01-1.0 mg.kg⁻¹) of LTHC compared to the control (time 0). At 15 minutes post-drug IOP was significantly different from control for the doses of 0.4 and 1.0 mg.kg⁻¹ LTHC.
(p<0.001). However at 30 minutes 0.1 (p<0.001), 0.4 (p<0.001) and 1.0 (p<0.001) mg.kg⁻¹ LTHC produced a significant decrease in IOP. The reduction in IOP seen with all doses of LTHC returned close to control values within 105 minutes of administration. Figure 3.4B shows the peak ΔIOP following i.t. LTHC administration. The mean (± S.E.M.) ΔIOP were 0.18±0.14, 0.73±0.3, 1.5±0.4, 2.1±0.35 and 2.33±0.27 mmHg for i.t. LTHC doses of 0.01, 0.05, 0.1, 0.4 and 1.0 mg.kg⁻¹ (n=4-8), respectively. The ED₅₀ for i.t. LTHC was 0.08 mg.kg⁻¹. The differences in mean ΔIOP between treatment groups were significant (p=0.004), with 1.0 (p<0.001), 0.4 (p<0.001), and 0.1 (p<0.05) mg.kg⁻¹ LTHC significantly different from the lowest dose of 0.01 mg.kg⁻¹ LTHC.

Figure 3.5A shows the effect of i.p. administration of LTHC (0.01-1.0 mg.kg⁻¹) on IOP. With the exception of the 1.0mg.kg⁻¹ dose all doses of LTHC tested failed to produce a drop in IOP by 30 minutes post-administration. However, at 45 minutes a significant reduction in IOP, compared to control (0 min), was seen with doses of LTHC of 0.2 (p<0.05), 0.4 (p<0.01) and 1.0 (p<0.001) mg.kg⁻¹. The decrease in IOP with all i.p. doses of LTHC returned to control values within 105 min. Figure 3.5B shows peak ΔIOP following i.p LTHC administration. The mean (± S.E.M.) ΔIOP were 0.075±0.15, 1.20±0.23, 1.52±0.23, 1.37±0.26 and 2.15±0.12 mmHg for i.p. LTHC doses of 0.01, 0.1, 0.2, 0.4 and 1 mg.kg⁻¹, respectively. The ED₅₀ for i.p. LTHC was 0.13 mg.kg⁻¹. There were significant differences in the mean ΔIOP for treatment groups 1.0 mg.kg⁻¹ (p<0.001), 0.4 mg.kg⁻¹ (p<0.01), and 0.2 mg.kg⁻¹ (p<0.01) compared with 0.1 and 0.01 mg.kg⁻¹ i.p. LTHC groups.
Figure 3.1  CB1Rs in ciliary epithelium and anterior ocular tissues.  (A) Product of RT-PCR reaction showing the presence of 500 bp band for CB1R mRNA in CE.  (B) Immunohistochemical staining of CB1R; strong labelling for CB1R in NPCE cells.  (C) Negative control (no primary antibody for CB1R used) with a section of the anterior portion of a rat eye, including the ciliary epithelium, showing negative CB1R staining.  Scale Bar = 50μm.
Figure 3.2  CB1Rs in HTM5 and NPCE cell lines (A) Product of RT-PCR reaction showing the presence of 500 bp band for CB1R mRNA in HTM5 (TM) and NPCE cell lines, as well as in rat retina (R) (positive control). (B) Presence of 53 kDa CB1R protein in HTM5 cell line, as determined by Western Blot analysis. (C) Presence of 250 kDa oligomeric form of CB1R protein in HTM5 cell line, determined by Western Blot analysis. (D) Immunocytochemical staining of CB1R in HTM5 cells. (E) Negative control (no primary antibody used) showing negative CB1R staining in HTM5 cells. Scale Bar = 20μm.
Figure 3.3 Effects of i.p. administration of MA on IOP in Brown Norway rats. The doses of 1.7 and 2.5 mg.kg$^{-1}$ were used in the experiments. The IOP-lowering effect of MA (2.5 mg.kg$^{-1}$) was blocked by pre-administration of SR141716A (2.5mg.kg$^{-1}$) i.p. Values represent the mean ± S.E.M. of 4-7 animals per group * (P<0.05), ** (P<0.01) and *** (P<0.001) significant differences between control (time 0) and drug-treated animals.
Figure 3.4  Dose-response relationship for the IOP-lowering effect of LTHC after i.t. administration. Doses of 0.01, 0.05, 0.1, 0.2 and 1.0 mg.kg⁻¹ were used in the experiment. Values represent the mean ± S.E.M. of 4-8 animals per group.  (B) Maximum Δ IOP after i.t. administration of different doses (0.01, 0.05, 0.1, 0.2 and 1.0 mg.kg⁻¹) of LTHC. * (P<0.05), ** (P<0.01) and *** (P<0.001) significant differences between control (time 0) and drug-treated animals.
Figure 3.5  (A) Dose-response relationship for the IOP-lowering effect of LTHC after i.p. administration. Doses of 0.01, 0.1, 0.2, 0.4 and 1.0 mg.kg\(^{-1}\) were used in the experiments. Values represent mean ± S.E.M. of 4-7 animals per group. (B) The peak change in IOP (Δ IOP) following i.p. administration of 0.01, 0.1, 0.2, 0.4 and 1.0 mg.kg\(^{-1}\) of LTHC. * (P<0.05), ** (P<0.01) and *** (P<0.001) significant differences between control (time 0) and drug-treated animals.
3.1.4 Synthetic Cannabinoid WIN55,212-2 Reduces IOP in Brown Norway Rats.

The aim of this study was to compare the IOP-lowering effect of LTHC to the synthetic cannabinoid agonist WIN55,212-2. Figure 3.6A shows the effect of the synthetic cannabinoid, WIN55,212-2 (0.017-17 mg.kg⁻¹) administered i.p. over a 120 minutes time-course. At doses of 0.017 and 0.17 mg.kg⁻¹ there was no change in IOP over the 120 minutes time-course as compared to the control (time 0) or vehicle-only group (p<0.05). Administration of 1.7 and 17 mg.kg⁻¹ of WIN55,212-2 produced a significant decrease in IOP (p<0.01) at 30 minutes after drug administration with 17 mg.kg⁻¹ WIN55,212-2 giving a significant reduction in IOP (p<0.001) within 15 min. The IOP-lowering effect of WIN55,212-2 returned to within control values between 90-120 minutes after drug administration. Figure 3.6B shows the peak ΔIOP following 0.017-17 mg.kg⁻¹ WIN55,212-2 doses. The mean (± S.E.M.) ΔIOP were 0.025±0.27, 0.20±0.32, 2.12±0.13, and 2.90±0.14 mmHg for i.p. administration of WIN55,212-2 doses 0.017 (n=4), 0.17 (n=4), 1.7 (n=6), and 17 mg.kg⁻¹ (n=4), respectively. The EC₅₀ for WIN55,212-2 was 1.0 mg.kg⁻¹. Compared with the i.p. WIN55,212-2 treatment groups at doses of 0.017 and 0.17 mg.kg⁻¹, significant differences were seen (p<0.001) in the mean ΔIOP for treatment groups at doses 1.7 and 17 mg.kg⁻¹. No significant differences between mean ΔIOP were observed between i.p. doses of 17 mg.kg⁻¹ WIN55,212-2 and i.p. or i.t. doses of 1.0 mg.kg⁻¹ LTHC (P>0.05). However, the ΔIOP seen with 17 mg.kg⁻¹ was significantly greater than those seen with 0.17 mg.kg⁻¹ WIN55,212-2 and 1.0 mg.kg⁻¹ of LTHC (p<0.001).
Figure 3.6  (A) Dose-response relationship for the IOP changes induced by i.p. administration of WIN55,212-2. Doses of 0.017, 0.17, 1.7 and 17 mg.kg$^{-1}$ were used in the experiment. Values represent the mean ± S.E. of 4-8 animals per group. (B) The peak change in IOP (Δ IOP) following i.p. administration of 0.017, 0.17, 1.7, 17 mg.kg$^{-1}$ of WIN55,212-2. *(P<0.05), ** (P<0.01) and *** (P<0.001) significant differences between control (time 0) and drug-treated animals.
3.1.5 LTHC and WIN55,212-2 Reduce IOP via Activation of CB1Rs

In order to determine whether the decrease in IOP by cannabinoid agonist is mediated by CB1R the antagonist/inverse agonist SR141716A was used. Figure 3.7 A and B demonstrate that the IOP-lowering effects of both LTHC and WIN55,212-2 are blocked by i.p. administration of the CB1R antagonist SR141716A. Following i.p. administration of 0.2 mg.kg⁻¹ LTHC the IOP was significantly lowered with a peak reduction from 20.4±0.32 to 18.9±0.168 mmHg at 45 minutes (Fig. 3.7A). In contrast, there was no significant difference in IOP at any time-point tested between animals treated with SR141716A only or LTHC plus SR141716A (p>0.05).

Intraperitoneal administration of 1.7 mg.kg⁻¹ of WIN55,212-2 (Fig. 3.7 B) produced a peak reduction of IOP from 20.17±0.25 to 18.05±0.22 mmHg at 30 minutes post administration. In contrast, there was no significant difference in IOP at any time-point between animals treated with SR141716A only or WIN55,212-2 plus SR141716A (p>0.05).

3.1.6 Co-Administration of LTHC and Timolol Does Not Produce an Enhanced Decrease in IOP in Brown Norway Rats

This study investigated whether the addition of the β-adrenergic blocker timolol, an agent which blocks the production of AH by CBE, will enhance the IOP lowering effect of LTHC and therefore will be useful in combination therapy for glaucoma. Figure 3.8 shows the effect of LTHC and timolol administered both individually and together on IOP in Brown Norway rats (n=4 for each group). Intraperitoneal administration of LTHC (0.2 mg.kg⁻¹) resulted in a significant reduction in IOP at 60, 75 and 90minutes after the
drug administration (p< 0.05). The peak ΔIOP of 1.725 ± 0.592 (mean ± SEM) occurred at 60 minutes following the LTHC administration. Topical administration of timolol alone to the right eye of each rat (0.05%; ~ 40μl) resulted in significant IOP-lowering effects at 60, 90 and 105 minutes after application (p< 0.05, p< 0.01, p< 0.05, respectively). The peak reduction in IOP with timolol occurred at 90 minutes after drug administration with the IOP decreasing from 19.5 ± 0.29 to 17.15 ± 0.38 mmHg (mean ± SEM). The peak ΔIOP (mean ± SEM) attained with the administration of timolol was 2.35 ± 0.202. There was a significant difference in IOP-lowering effects after the administration of both i.p. LTHC and topical application of timolol at 30 (p< 0.05), 45 (p< 0.01), 60 (p<0.001), 75 (p< 0.01), 90 (p<0.01) and 105 minutes (p< 0.01) as compared to control (time 0). Peak reductions in IOP with both of these drugs were seen at 60 minutes from 20.25 ± 0.51 to 17.8 ± 0.35 mmHg (mean ± SEM), the mean (± SEM) ΔIOP was 2.45 ± 0.651. There was a significant difference between LTHC treated group and timolol treated group at 90, 105, and 120 minutes after the drug administrations, with (p< 0.05). The IOP-reduction attained with timolol alone was longer lasting than that produced by LTHC.

The difference in the mean values of IOP measurements across specific time points (0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes) between either LTHC group or timolol group, and LTHC and timolol administered together were not great enough (p= 0.05) to be statistically significant.
Figure 3.7  Antagonism of IOP-lowering effects of LTHC and WIN55,212-2 by i.p. administration of SR141716A. Doses of 2.5 mg.kg$^{-1}$ SR141716A were administered 30 minutes prior to the administration of (A) 0.2 mg.kg$^{-1}$ LTHC, (B) 1.7 mg.kg$^{-1}$ WIN55,212-2. * (P<0.05), ** (P<0.01) and *** (P<0.001) significant differences between WIN55,212-2-treated group and SR141716A or SR141716A+WIN55,212-2 groups.
Figure 3.8  Changes in IOP in Brown Norway eyes after administration of LTHC and timolol. The i.p. LTHC (0.2 mg.kg\(^{-1}\)) and topical timolol (0.05%, ~ 40\(\mu\)l) were administered alone, or in combination with each other. Values represent the mean ± S.E.M. of 4 animals per group *(P<0.05), **(P<0.01) and ***(P<0.001) significant differences between control (time 0) and drug-treated animals.
3.1.7 Co-Administration of LTHC and Latanoprost Does Not Produce an Enhanced Decrease in IOP in Brown Norway Rats

Prostaglandin analog latanoprost, a common drug used in the treatment of glaucoma, was co-administered with LTHC to Brown Norway rats. The IOP lowering effects of latanoprost and LTHC by both combined and individual administration were compared.

Figure 3.9 shows the effect of LTHC (0.2 mg.kg⁻¹) and the prostaglandin analog latanoprost (0.005%, ~ 40μl volume) on IOP in Brown Norway rats (n= 6 for each group). Each drug was administered individually and then again administered together. LTHC administered alone significantly reduced IOP at 45 minutes post-administration, the IOP decreasing from 20.45 ± 0.21 to 19.08 ± 0.16 mmHg (mean ± SEM), (p< 0.01). The mean ΔIOP (± SEM) was 1.367 ± 0.187 mmHg. A significant reduction in IOP produced by administration of latanoprost alone was first seen at 60 minutes after drug administration and persisted throughout the whole experiment (p< 0.05). The peak IOP-reduction with this drug was observed at 120 minutes, with IOP decreasing from 20.4 ± 0.21 to 19.2 ± 0.17 mmHg (mean ± SEM), (p< 0.01). The mean ΔIOP (± SEM) was 1.2 ± 0.211 mmHg. The concomitant administration of both, LTHC and latanoprost resulted in significant IOP-lowering effect occurring at 30 minutes, 15 minutes earlier than IOP-lowering effect of each drug administered individually (p< 0.05). But there was no apparent additive effect for the combined therapy. However, the intraocular hypotensive effect of the drug combination persisted up to 120 minutes (end of the experiment), with the peak IOP-decrease at 60 minutes, from 21.2 ± 0.4 to 19.15 ± 0.23 (mean ± SEM), (p< 0.001). The mean ΔIOP (± SEM) was 2.05 ± 0.284 mmHg.
Figure 3.9  Effects of i.p. administered LTHC (0.2 mg.kg\(^{-1}\)) and topically administered latanoprost (0.005%, ~ 40μl), both alone, and in combination, on IOP in Brown Norway rats. Values represent the mean ± S.E.M. of 4-8 animals per group *(P<0.05), **(P<0.01) and ***(P<0.001) significant differences between control (time 0) and drug-treated animals.
3.1.8 The Concentration of Δ9-THC in Rat Plasma, After i.t. LTHC Administration

The Gas Chromatography/Mass Spectometry (GC/MS) analysis of plasma Δ9-THC concentration after i.t. LTHC administration in Brown Norway Rats (n= 2-3 per each time point) was carried out to determine the levels of the drug for the period of 240 minutes post-drug administration. High variability in plasma levels of Δ9-THC was observed in samples obtained for each of the time points. The peak in the Δ9-THC concentration (mean ± SEM) of Δ9-THC occurred at 15 minutes (12.5 ± 3.7 ng/ml), and slowly declined to 1.9 ± 0.5 ng/ml, 120 minutes post drug delivery (data not shown).

3.1.9 The Concentration of Δ9-THC in Rabbit Ocular Tissues, After Topical, i.t. and i.v. LTHC Delivery

Following topical delivery of LTHC (30 µg or 60 µg) to the right eye of each rabbit and the bioavailability of Δ9-THC in ocular tissues (cornea, CB, retina, lens and VH) was determined by GC/MS analysis (DRDC, Toronto, Ontario).

From the five tissue samples dissected from each eye (cornea, lens, CE, retina and vitreous humor (VH)) (n= 2-3) (Figure 3.10) and treated with 30 µg of LTHC, the peak concentration of free Δ9-THC were as followed: 11838.2 ng/g in the cornea (Figure 3.10A), 20.7 ng/g in the CB (Figure 3.10B), and 154 ng/g in the retina (Figure 3.10C). The peak concentration of free Δ9-THC in VH and lens were 21.0 ng/g and 3.9 ng/g, respectively (data not shown). The tissues obtained from eyes treated with 60 µg of LTHC showed the similar distribution of free Δ9-THC as seen with 30 µg LTHC application (Figure 3.11). The mean peak values (n= 2-3) for Δ9-THC concentrations
were as followed: 5397.4 ng/g in cornea (Figure 3.11A), 46.95 ng/g in the CB (Figure 3.11B), and 80.63 ng/g in the retina (Figure 3.11C). Free Δ9-THC was also detected in the VH and lens, with peak concentrations: 7.23 ng/g and 2.2 ng/g, respectively. In the rabbit contralateral (left) eyes, with no LTHC application, small amounts of Δ9-THC were detected.

The ocular tissue concentrations of free Δ9-THC following i.t. delivery of LTHC (150 μg; 250 μl volume) to lungs of each rabbit was analyzed by GC/MS analysis. From five tissue samples dissected from each eye (cornea, CB, retina, lens and VH) (n = 2-3) the peak averaged concentrations of Δ9-THC occurred 15 minutes following LTHC administration and were as followed: 1.05 ng/g in the cornea (Figure 3.12A), 9.55 ng/g in the CB (Figure 3.12B), and 5.05 ng/g in the retina (Figure 3.12C). Low concentration of Δ9-THC (0.05 ng/g) was also present in VH (data not shown). No detectable amount of Δ9-THC was present in lens (data not shown).

The ocular tissues concentrations of free Δ9-THC following i.v. delivery to the marginal ear vein of each rabbit of LTHC (150 μg; 250 μl volume) were determined by GC/MS. From five tissue samples dissected from each eye (cornea, CB, retina, VH and lens) (n=2-3) the peak averaged concentrations of free Δ9-THC were as followed: 4.35 ng/g in the cornea (Figure 3.13A), 1.7 ng/g in the CB (Figure 3.13B), and 3.85 ng/g in the retina (Figure 3.13C). The low levels of free Δ9-THC were also detected in lens (0.15 ng/g) (data not shown), with no detectable amount of Δ9-THC present in VH (data not shown).
3.1.10 The Concentration of Δ9-THC in Rabbit Plasma After i.t. and i.v. LTHC Delivery

The GC/MS analysis of plasma Δ9-THC concentration after i.t. and i.v. LTHC administrations in New Zealand White rabbits was carried out to determine the bioavailability of the drug for the period of 30 minutes post-drug administration. Figure 3.14A shows the plasma concentration of Δ9-THC following i.t. LTHC administration. The peak in Δ9-THC concentration (80 ± 12.2 ng/ml) occurred 3 minutes, following the drug administration and gradually decreased to 3.0 ng/ml, 30 minutes after administration. Figure 3.14B shows the plasma concentration of Δ9-THC following i.v. LTHC administration over the period of 30 min. The peak in Δ9-THC concentration (42.9 ± 4.2 ng/ml) occurred 3 min, following the drug administration and gradually declined to 3.1 ng/ml, 30 minutes after administration.

3.1.11 LTHC Reduces IOP in New Zealand White Rabbits.

The pharmacodynamic profile for LTHC administered by various routes to New Zealand White rabbits was examined by studying the effect of LTHC on IOP measurements.

Figure 3.15A shows the effect of topical administration of two doses, 30 μg and 60 μg, of LTHC (n= 3 for each group) to right eye of rabbits. The highest drop in IOP (mean ± SEM), with topical application of 60 μg of LTHC, was observed 120 minutes post-drug administration from 20.8 ± 0.3 to 19.5 ± 0.3 mmHg. With 30 μg dose of LTHC the peak in IOP drop occurred at 30 minutes post-drug administration, from 20.56 ± 0.25 to 19.2 ± 0.87 mmHg. The left eye served as a control. Although a decrease in IOP with
both doses of LTHC was observed, it was not significant, as compared to control (time 0) (p<0.05).

Figure 3.15B shows the effect of i.t. administration of 150 μg of LTHC (n= 3) on IOP in New Zealand White rabbits, evaluated over the period of 240 minutes after administration. The highest drop in IOP with LTHC was observed at 30 minutes with IOP decreasing from 20.3 ± 0.1 mmHg to 18.8 ± 0.3 mmHg. The mean (± SEM) ΔIOP was 1.5 ± 0.3 mmHg. The IOP-lowering effect of LTHC was statistically significant at both 15 and 30 minutes following drug administration (p< 0.05).

Figure 3.15C shows the effect of i.v. administration of 150 μg of LTHC (n= 3) to New Zealand White rabbits. The highest drop in IOP with i.v. LTHC was observed at 15 minutes with IOP decreasing from 23.3 ± 1.1 mmHg to 21.5 ± 0.7 mm Hg. The difference in IOP-lowering effect across the different time points was not statistically significant (p< 0.05).

3.1.12 LTHC Administration Does Not Affect Pupil Diameter in New Zealand White Rabbits.

The effect of topical LTHC administration on changes in pupil diameter, which may affect AH outflow facility and IOP, was investigated in New Zealand White rabbits across 240 minutes period. Figure 3.16 show that the unilateral application of 60 μg (150 μl) LTHC to the right eye of each rabbit did not affect pupil diameter (Figure 3.16) as compared to liposomal control.
Figure 3.10  Average concentration of Δ9-THC (ng/g) in ocular tissues after topical administration of 30 µg LTHC to the right eyes of New Zealand White rabbits; the left eye served as the non-drug treated control. Values represent mean of 2-3 tissue samples per each time point. (A) Δ9-THC concentration in cornea. (B) Δ9-THC concentration in ciliary body (CB). (C) Δ9-THC concentration in retina. THC concentrations were determined by GC/MS analysis.
Figure 3.11  Average concentrations of Δ9-THC (ng/g) in ocular tissues after topical administration of 60 μg LTHC to right eye of New Zealand White rabbits; the left eye served as the non-drug treated control. Values represent mean of 2-3 tissue samples per each time point. (A) Δ9-THC concentration in cornea. (B) Δ9-THC concentration in ciliary body (CB). (C) Δ9-THC concentration in retina. Δ9-THC concentrations were determined by GC/MS analysis.
Figure 3.12  Average concentration of Δ9-THC (ng/g) in ocular tissues from New Zealand White rabbits, following i.t. administration of 150 μg of LTHC, determined by GC/MS analysis. Values represent the mean of 2-3 tissue samples per each time point. (A) Δ9-THC concentration in cornea. (B) Δ9-THC concentration in ciliary body (CB). (C) Δ9-THC concentration in retina.
A

B

C
Figure 3.13  Average concentration of Δ9-THC in ocular tissues of New Zealand White rabbits following i.v. administration of 150 μg of LTHC. Values represent the mean of 2-3 tissue samples per each time point, as determined by GC/MS.  (A) Δ9-THC concentration in cornea.  (B) Δ9-THC concentration in ciliary body (CB).  (C) Δ9-THC concentration in retina.
Figure 3.14  Plasma Δ9-THC concentration following i.t. and i.v. LTHC administration (ng/ml) (mean ± SEM). (A) i.t. LTHC (150μg) administration and (B) i.v. LTHC administration (150 μg) to New Zealand White rabbits. Values represent the mean of 2-3 tissue samples per each time point.
A

B
Figure 3.15  Effect of LTHC on IOP in New Zealand White rabbits, following:  (A) topical administration of 30 μg and 60 μg LTHC (B) i.t. administration of 150 μg LTHC (C) i.p. administration of 150 μg of LTHC. Values represent mean ± S.E.M. of 2-3 animals per group. * (p<0.05) significant differences between control (time 0) and drug-treated animals.
Figure 3.16  The effect of topical administration of LTHC (60 μg) on pupil diameter in New Zealand White rabbits. Values represent the mean ± SEM of 3 animals. No significant change in pupil diameter was found following administration of the drug as compared to control.
3.2 CB1R and M3R Agonists Modulate TM Contractility

3.2.1 M3Rs, SMA and Vinculin are Expressed in TM Cells

In order to determine the presence of M3Rs, a target for muscarinic agonists such as carbachol, in HTM5 cells, immunocytochemical staining was used in this experiment. In addition, TM cells were also stained for structural proteins including smooth muscle actin (SMA) and vinculin (a protein that is involved in the attachment of actin-based microfilaments to the plasma membrane) in order to confirm their expression in HTM5 cell line. Figure 3.17A shows the positive staining for M3R, which was determined with a polyclonal antibody directed against this receptor. Figure 3.17B shows the staining of α-SMA, while Figure 3.17C shows the staining of Vinculin.

3.2.2 CB1R and M3R Agonists Increase MLC Phosphorylation in TM Cells

Muscarinic agonists have been reported to increase TM contractility in isolated Bovine strips (Thieme et al., 2000), as well as in TM obtained from monkey eyes (Fukiage et al., 2001). The contractility of TM is reported to be regulated by the phosphorylation/dephosphorylation of the myosin light chain in the Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent manner (Fukiage et al., 2001; Thieme et al., 1999; 2000). Therefore, we tested the M3R agonist Carbachol as well as the CB1R agonist WIN55,212 to determine the effects of these ligands on MLC phosphorylation in human TM cells.

Immunoblot analysis (n=2) with antibody directed against pMLC protein showed an increase in MLC phosphorylation in HTM5 cells after 5 and 15 minutes exposure to 100 μM Carbachol (Figure 3.18A&B) (n=2). Lower concentrations of Carbachol (0.1-10 μM) had no affect on the MLC phosphorylation as compared to the control (no drug).
Figure 3.17  Immunocytochemical staining of cultured HTM5 cells for (A) M3Rs (B) Structural protein smooth muscle actin (α-SMA) and (C) Vinculin containing focal adhesions. Scale Bar = 20μm.
Figure 3.18  Effect of the MR agonist, carbachol, on MLC phosphorylation in HTM5 cell line. (A) Representative immunoblot showing the pMLC in cultured HTM5 treated with 0.1-100μM Carbachol. (B) Percentage change in MLC phosphorylation with 0.1-100μM Carbachol, as compared to control (represented as 100%) (n=2).
We next examined the effects of WIN55,212-2 (0.01-100 μM) on MLC phosphorylation in serum-starved HTM5 cells, following their 5 and 15 minutes exposure to this ligand. WIN55,212-2 (0.1-100μM) increases MLC phosphorylation in a dose dependent manner. The highest increase in MLC phosphorylation was seen at 5 minutes with 10 and 100 μM WIN55,212-2 (p<0.01) and was completely inhibited by pre-treatment of cells with 10 μM of CB1R antagonist SR141716A (Figure 3.19A&B). However, a longer agonist exposure (15 min) with WIN55,212-2 in contrast resulted in decreased levels in pMLC as compared to control (data not shown).

3.2.3 Both CB1R and M3R Activation Alter TM Cells Morphology

The phosphorylation of MLC in TM has been reported to results in TM cell contractility, which is associated with change in actin architecture and alterations in cell morphology (Wettschureck and Offermanns, 2002; Somlyo and Somlyo, 2000; Totsukawa et al., 2000). In our study the exposure of TM cells to muscarinic agonist carbachol and cannabinoid agonist WIN55,212-2 increased pMLC (see section 3.2.1).

Therefore, we investigated if the exposure of TM cells to either Carbachol or WIN55,212-2 would alter cell TM morphology. Treatment of TM cells with 1-10 μM of WIN55,212-2 resulted in structural changes in actin architecture and alterations in cell morphology as revealed by phalloidin staining which labels the polymeric and oligomeric forms of actin. While vehicle-treated cells exhibited a "normal" appearance (Figure 3.20A) a 30 sec- 5 minutes exposure to 10 μM WIN55,212-2 resulted in a change in cell morphology characterized by a narrowing or thinning of the cell body (Figure 3.20B) producing an elongated shape. Conversely, a 10 minute treatment with WIN55,212-2
resulted in a more spread out effect and overall circular appearance of the cell body with the development of numerous cytoplasmic projections (Figure 3.20C).

The treatment of HTM5 cells with Carbachol (1-100 μM) also induced dynamic changes in cell morphology. After 0.5 to 5 minute treatments with carbachol TM cells become narrower, and developed punctuate structures and focal adhesions. In addition stress fibres oriented primarily parallel to the long axes of cells were observed (Figure 3.21B). After treatment of cells for 10 minutes with the same concentration of Carbachol cells appeared more rounded, however altered actin architecture and focal adhesion points were still evident (Figure 3.21C).

Figure 3.22 shows the morphological changes in HTM5 cells after treatment of these cells with KCl (1-100 mM), which depolarizes cell membranes and increases intracellular Ca²⁺. Significant changes in TM cells’ morphology were evident as early as 1 minute after the treatment with KCl (Figure 3.22B) and persisted for at least 10 minutes (Figure 3.22C). As seen with carbachol, KCl treatment induced contractility of these cells as well as development of focal adhesions and stress fibres.

3.2.4 Effects of WIN55,212-2 and Cytochalasin D on HTM5 CSK Stiffness

Figure 3.23 shows the preparation of HTM5 cells with attached ferromagnetic beads, which were used in OMTC experiments and allowed to evaluate the effect of the WIN55,212-2 and Cytochalasin D, which disrupts actin microfilaments, on TM cell CSK stiffness.

OMTC analysis showed that the CSK stiffness of HTM5 cells is decreased with an application of Cytochalasin D, (Figure 3.26A). The analysis of the effect of 10 μM
WIN55,212-2 on HTM5 CSK stiffness, assessed with OMTC technique, showed that the drug does not affect cell stiffness in the first 30 seconds after the drug application. However, a significant increase in HTM5 cells' stiffness was evident after 90 sec following WIN55,212-2 addition (Figure 3.26B). The analysis of the data by paired T-Test showed that the difference in cell stiffness was significant with p<0.001 (***).
Figure 3.19  Effect of WIN55,212-2 on MLC phosphorylation in HTM5 cell line. (A) Representative immunoblot of pMLC in cultured HTM5 cells treated with 10 μM WIN55,212-2.  (B) The percentage of MLC phosphorylation, as determined by densitometry analysis, with 0.01-100 μM WIN55,212-2, as compared to control (n=3). The increase in MLC phosphorylation with WIN55,212-2 was inhibited by pre-treatment of cells with 10 μM SR141617A, the selective CB1R antagonist/inverse agonist. ** (p< 0.01) significant difference between control (no drug) and drug treated groups.
Figure 3.20  FITC-phalloidin staining of actin filament in HTM5 cells, treated with WIN55,212-2.  (A) Vehicle treated cells.  (B) Cells treated for 3 minutes with 10 μM WIN55,212-2.  (C) Cells treated for 10 minutes treatment with 10 μM WIN55,212-2 as compared to control. Scale bar = 20 μm.
Figure 3.21 Staining of actin filament, with FITC-phalloidin, in HTM5 cells treated with carbachol. (A) Vehicle treated HTM5 cells. (B) Cells treated for 3 minutes with carbachol (100µM), white arrows focal stress adhesions. (C) Cells treated for 10 minutes with carbachol (100 µM). Scale Bar = 20 µM.
Figure 3.22  Staining of actin filaments, with FITC-phalloidin, in HTM5 cells treated with KCl.  (A) Vehicle-treated HTM5 cells.  (B) Cells treated for 3 minutes with KCl (100 mM).  (C) Cells treated for 10 minutes with KCl (100 mM).  White arrows label focal stress adhesion.  Scale bar = 20 μM.
Figure 3.23 Changes in HTM5 cell stiffness in response to CB1R activation or exposure of cells to the CSK active agent, Cytochalasin D. (A) Phase picture of HTM5 cells with attached ferromagnetic beads. (B) Phalloidin staining of HTM5 cells with attached ferromagnetic beads. (C) Enlarged view of cell shown in (B). (D) Alternation in cell stiffness following treatment with Cytochalasin D (CTCh) (1 μM). (E) WIN55,212-2 (WIN55,212-2; 10 μM) cell stiffness was measured in the absence (0 sec) and presence (30 or 90 sec) of a drug. Values represent mean ± SEM. *** Significant difference (p<0.001) was assessed with paired t-test.
CHAPTER 4

Discussion
This thesis investigated the effectiveness of liposomal encapsulation of Δ9-THC as a potential drug delivery system for cannabinoid compounds. Specifically, my research investigated the \textit{in vivo} action of LTHC and other cannabinoid drugs on IOP in the eye. This allowed a non-subjective pharmacological assessment of the LTHC acting at receptors to give a quantitative response. The tissue and plasma levels of Δ9-THC after various routes of LTHC administration were also evaluated, in order to determine whether liposomal encapsulation of Δ9-THC could provide sufficient bioavailability of LTHC to account for the observed physiological response. In addition, experiments \textit{in vitro} further examined the actions of cannabinoid ligands at receptors in tissue of the AH outflow pathway, such as trabecular meshwork. This tissue is responsible for modulating of AH outflow and is a potential target for glaucoma drugs.

My results showed that LTHC preparations administered by different routes result in therapeutic levels of Δ9-THC in both ocular tissues and plasma and produce a decrease in IOP in both rat and rabbit. These effects occurred primarily via activation of CB1Rs. In addition, my experiments demonstrated that cannabinoid agonists modulate TM cell morphology and alter cell-signalling pathways that regulate CSK in these cells.

4.1 Liposomes as a Drug Delivery System for Cannabinoids

The \textit{in vivo} experiments performed in our study evaluated the ability of a liposome-encapsulated preparation of Δ9-THC, LTHC, to deliver a sufficient dose of Δ9-THC via the lungs in rats in order to produce a desired pharmacodynamic effect. The hypothesis of this study was that liposome encapsulation of Δ9-THC would increase the
bioavailability of the drug allowing it to reach ocular tissues and that the intratracheal route of administration would be superior to that of parenteral administration via i.p. injection. Our results showed that i.t. LTHC delivery resulted in a more rapid decrease in IOP in the rat eye, most likely due to rapid absorption of the drug from the alveoli into systemic circulation. Estimates of EC$_{50}$ suggested that i.t. LTHC was of similar potency as i.p. LTHC, with the maximal efficacy comparable between both routes of LTHC administration.

The IOP-lowering effect of LTHC following i.p. administration was also compared to that of synthetic cannabinoid agonist, WIN55,212-2. This study showed that while WIN55,212-2 decreased IOP after i.p. administration with a higher maximal efficacy than LTHC, the potency of LTHC, delivered by either i.p. or i.t. routes, to decrease IOP was greater than WIN55,212-2.

With regard to the duration of action, the IOP-lowering effect of both LTHC and WIN55,212-2 returned to base-line levels approximately 1.5-2 hrs after drug administration. Δ9-THC is a highly lipophilic drug with a large volume of distribution. An average estimated volume of 3.4 l.kg$^{-1}$ (or 236 l for a 70 kg subject) has been reported (for review see Grotenhermen, 2003). The short duration of LTHC drug effect can, in part, be explained by the rapid redistribution of Δ9-THC to other tissues. At this point it should be mentioned that the liposomes used for the encapsulation of Δ9-THC were unilamellar vesicles of a uniform size (368 ± 42 nm from the Coulter N4SD particle-size analyzer) and therefore it is likely that the Δ9-THC was released at the same rate from all liposomes. Since drug release in vivo is influenced by size and lipid composition, the smaller sized liposomes with larger curvature and looser lipid packing tend to release
drug more rapidly whereas larger liposomes composed of cholesterol and rigid phospholipids, such as hydrogenated phosphatidylcholine, distearoylphosphatidylcholine and hydrogenated phosphatidylinositol, exhibit less drug leakage and a more protracted drug release time (for review see, Torchilin, 2005; Nagayasu et al., 1999). We anticipate that the duration of action of LTHC can be prolonged by a revised lipid formulation including a multilamellar liposomal preparation technique producing a mixed population of liposome sizes. It may also be possible to increase the circulation time of liposomes by incorporation of distearoylglycerophosphoethanolamine methoxylpolyethyleneglycol 2000 (DSPE-MPEG), which reduces the removal of liposomes by the reticuloendothelial system (Allen et al., 2000).

Our results provide evidence that inhalation exposure to aerosolized liposome-encapsulated cannabinoids may be a superior means to deliver cannabinoid drugs for therapeutic use. Although relatively few studies have evaluated the use of aerosolized cannabinoids for drug delivery, several early studies examined the delivery of aerosolized Δ9-THC to human subjects by either a metered-dose inhaler (Tashkin et al., 1976) or a nebulizer (Vachon et al., 1976) for bronchodilation. Although inhalation delivery of Δ9-THC produced effective bronchodilation in normal subjects, chest discomfort and irritation precipitating cough was also noted and, in some asthmatics, bronchoconstriction as opposed to bronchodilation was seen (Tashkin et al., 1977). It was suggested that the local irritant effects might be due to the large particle size of the Δ9-THC aerosol or to the propylene glycol vehicle used in the nebulizer delivery (for review see Tashkin et al., 2002). However, for liposome-encapsulated drug delivery, the liposome phospholipid bilayer acts both as a permeable barrier to entrap drugs and provide a controlled and
sustained release system and also provides a means to protect local tissues by encapsulating an irritating or toxic drug (Banerjee, 2004). In humans, liposome-encapsulated orciprenaline and salbutamol into the respiratory system have been well tolerated and have been demonstrated to provide sustained bronchodilation in patients with chronic obstructive lung disease (Fielding, 1991). Furthermore, no adverse effects have been associated with inhalation of liposomes in healthy human volunteers (Hung et al., 1995; Thomas et al., 1991). In animal models, acute and chronic liposomal drug delivery did not adversely affect pulmonary histopathology or function (Oyarzun et al., 1980; Myers et al., 1993).

While this represents the first study to look at liposome-encapsulated Δ9-THC for inhalation delivery in rodents, aerosolized cannabinoids, including Δ9-THC, have been examined in mice. Lichtman et al. (2000) used a Small Particle Aerosol Generator (SPAG) nebulizer to generate an aerosol with an output of 0.154 mg/l of aerosolized Δ9-THC with particles less than 5.0 μm in diameter. In this study, Δ9-THC was formulated in a vehicle consisting of 1:1:18 ethanol/emulphor/saline. Inhalation exposure of the Δ9-THC aerosol in mice was able to elicit antinociceptive effects that were dependent on dose and time of exposure. Onset of antinociceptive effects, with an estimated 1.8 mg/kg of Δ9-THC occurred within 5 minutes, lasted approximately 40 minutes and were blocked by the CB1R antagonist SR141716A. In our study, intratracheal delivery of a dose of LTHC of 0.4 to 1.0 mg/kg produced a significant reduction in IOP with an EC₅₀ of 0.08 mg/kg.

Pharmacological studies examining possible alternate delivery systems to smoking for Δ9-THC in humans and animals have primarily examined oral, rectal and
topical formulations. Synthetic Δ9-THC (Marinol®) has been used to relieve nausea and vomiting related to cancer chemotherapy and to stimulate appetite in patients with acquired immunodeficiency disease (AIDS) (Rosen, 1995). However when taken orally, Δ9-THC undergoes significant hepatic first pass metabolism resulting in erratic and unpredictable absorption and bioavailability (<15%) and a delayed onset of action (Lemberger et al., 1973; Wall and Perez-Reyes, 1981; for review see, Grotenhermen, 2003). While rectal application can avoid the hepatic first pass effect with higher bioavailability than oral administration, bioavailability via this route varied substantially with different formulations (Brenneisen et al., 1996). Most ophthalmic studies of topical Δ9-THC and cannabinoid drugs in humans with glaucoma and in animal models have employed the use of vehicles such as light mineral oil (Chiang et al., 1983), cyclodextrin or hydroxypropylmethylcellulose (Green and Kearse, 2000; Porcella et al., 2001; Laine et al., 2003). Despite an IOP-lowering effect, topical application to the cornea has resulted in limited bioavailability and in some cases ocular irritation and toxicity (Green and Kearse, 2000; for review see, Järvinen et al., 2002; Grotenhermen, 2003). In our experiments neither route of LTHC administration to rat or rabbit produced any observed side effects. The topical administration of LTHC did not cause any observed irritation to eye tissue. The liposomal encapsulation of Δ9-THC allowed for lower doses of drug to be used, but were sufficient to produced drop in IOP. On the other hand, i.p. injection of the highest dose (17 mg/kg) of WIN55-212,2 (which resulted in the comparable IOP drop to the one obtained with the highest dose of LTHC (1mg/kg)) complete inhibited the motor behaviour in rat, an effect which persisted for approximately one hour.
We also investigated the possible additive effect of LTHC administered in combination with either the β-adrenergic blocker timolol or prostaglandin analogue latanoprost. The combination of drugs that target an AH production and outflow pathway such as timolol and latanoprost are used in the approximately one third of all patients with glaucoma who are unable to achieve satisfactory IOP-lowering effect with either timolol or latanoprost, alone. Our experiments showed that the concomitant administration of LTHC with either agent did not produce a significant additive IOP-lowering effect. However, the IOP-lowering effect of LTHC administered in combination with either timolol or latanoprost occurred more rapidly than with any of the drugs administered alone. It is important to mention that in this pilot experiment, only one dose (0.2 mg.kg⁻¹) of LTHC was used. It is possible that the co-administration of higher doses of LTHC with latanoprost or timolol may produce an improved and additive IOP hypotensive effect and this need to be addressed in future experiments.

While the potential for other systemic side effects such as the reduction in systemic blood pressure and psychomotor effects may argue against the use of Δ9-THC as a chronic anti-glaucoma drug. It is possible that lower doses of LTHC given via an inhalation route or the use of a liposome-encapsulated non-psychotropic cannabinoid component of marijuana, cannabidiol (CBD), or the synthetic nonpsychotropic cannabinoid, such as HU-211, may be useful therapies for the treatment of glaucoma and other chronic retinal diseases. One study in primates has shown that topical application of WIN55,212-2, a CB1R agonist, is effective in lowering IOP in monkey with experimentally-induced glaucoma. Another finding reported that intravitreal injections of CBD and Δ9-THC in rats protect retinal neurons from glutamate-induce excitotoxicity
(El-Remessy et al., 2003). Therefore, formulation of a preparation such as liposomal encapsulation of non-psychoactive ('lacking' side effects) cannabinoids that would target retina and tissues that regulate AH flow may provide both on IOP decrease and neuroprotection in treatment of glaucoma.

Our studies with LTHC indicate that inhaled liposome-encapsulated cannabinoids are an effective way to deliver pharmacologically active measured doses of cannabinoids. With the emerging impetus to develop cannabinoids for therapeutic use in chronic neurodegenerative conditions, the use of liposome-capsulation may provide an effective formulation for these highly lipophylic drugs, thus allowing alternative drug delivery routes to be employed. While our study compared inhaled versus intraperitoneal routes of LTHC, it is possible that topical application of LTHC will also prove to be effective with better corneal penetration, less toxicity and longer drug half-life than previously tested formulations. The availability of alternative delivery systems for cannabinoid drugs may lead to further utility of these drugs, as well as to the development of novel cannabinoids in the treatment of chronic eye disease.

4.2 The Bioavailability of Δ9-THC in Ocular Tissues

Our pharmacokinetic results, obtained from GC/MS analysis of rabbit ocular tissues showed that all routes of LTHC administration (i.t., i.v., topical) resulted in the presence of pharmacologically relevant (Heishman, 1990) amounts of Δ9-THC in all ocular tissues analyzed.

After topical delivery of LTHC (30 and 60 μg), Δ9-THC concentrations in ocular tissues were evaluated over the period of 240 minutes and were correlated to effect of the
drug on IOP. In the CB, a structure that plays an essential role in AH production, the peak in Δ9-THC concentration occurred 120 minutes after LTHC administration. Yet, the peak in IOP-lowering effect with both doses of the drug was observed 45 minutes after application of the drug to the eye. At that time point a sufficient amount of Δ9-THC was present in the CB to produce an IOP-lowering effect, therefore it would be expected that the effect of Δ9-THC would persist for a longer period of time. However, high concentrations of Δ9-THC in ocular tissues do not necessary reflect the presence of the drug at the site of receptors. Furthermore, prolonged exposure to cannabinoids may result in down regulation or desensitization of receptors, and therefore may diminish the physiological effect(s). At this point, it should be mentioned that in the contralateral (control) eyes, ‘trace’ amounts of Δ9-THC were also detected. This suggests that Δ9-THC enters the systemic circulation, most likely through the nasal mucosa and the ocular conjunctiva (Urtti and Salminen, 1993).

After i.t. and i.v. LTHC delivery to New Zealand White rabbits, the highest Δ9-THC concentrations were found in highly vascularized tissues, including CB and retina. In the avascular tissues, including lens and cornea, the concentration of Δ9-THC was relatively low. Although, following i.t. LTHC administration in rabbit, blood and tissue samples were collected over the period of 240 minutes, the concentrations of Δ9-THC in these samples were evaluated for only the first 30 minutes following drug delivery. With regards to plasma levels of Δ9-THC, both i.t. and i.v. administration resulted in comparable pharmacokinetic profiles, with the peak plasma concentration 3 minutes after deliver. The concentrations of the Δ9-THC in plasma following i.t. delivery was higher (80.0 ± 12.2 ng/ml (mean ± SEM)) as compared to i.v. administration (42.9 ± 4.2 ng/ml)
(mean ± SEM)). This difference can be explained by the fact that following i.v. administration, liposomes are rapidly removed from blood by the mononuclear phagocyte system, including the Kuffler cells of the liver and the fixed macrophages of the spleen (Allen, 1983). On the other hand, pulmonary delivery of liposome-encapsulated drugs have been shown to result in better retention of the preparations in the lungs and provided prolong systemic absorption and higher bioavailability of the drug. These effects were demonstrated with pulmonary inhalation of liposome-encapsulated sodium cromoglycate in human volunteers (Taylor et al., 1989), liposome-encapsulated salbutamol and orciprenaline (Fielding, 1991) and liposome encapsulated fentanyl (Hung et al., 1995, 1997).

The inconsistency between plasma or tissue levels and observed physiological effects is not recent, and has been reported with different routes of Δ⁹-THC administration by other investigators (Huestis, 1999; Perez-Reyes, 1999; Agurell et al., 1986). The most evident discrepancy between Δ⁹-THC and its effect are observed after oral administration. For example, ingestion of a 20 mg Δ⁹-THC capsule results in the maximum Δ⁹-THC pleasant sensory perception (PSP) 4 hours after administration, while the plasma concentration is already decreasing (Agurell et al., 1986). A similar phenomenon is observed with inhalation of marijuana cigarettes, where maximum PSP lags behind the maximum Δ⁹-THC plasma concentration (Agurell et al., 1986). Interestingly, the same study showed that significant concentrations of Δ⁹-THC are still present in plasma 240 minutes after administration, while PSP is absent. The discrepancies between tissue and plasma levels of Δ⁹-THC can perhaps be explained by specific pharmacokinetics for this particular drug. The immediate uptake or deposition of
free drug in neutral fat depots limits the bioavailability for Δ9-THC for target tissues (Huestis, 1999). For example, five days after administration of Δ9-THC by inhalation, 50% of unmetabolized drug was still present in fat depot (Garrett et al., 1979). Finally, upon its release from liposomes, Δ9-THC like many other lipophilic molecules binds to plasma proteins (Garrett and Hunt, 1974). Therefore its recovery from plasma may be related to the release of the protein-bound Δ9-THC.

4.3 Relevance of Animal Models to Glaucoma

To study and understand the pathology of glaucoma and to develop new treatments for this disease different experimental approaches have been employed. The aim of these approaches is to achieve simple, inexpensive and reproducible models of the disease, but most importantly to develop a model as similar to human glaucoma in its pathology as possible. The most common models used for studying the disease rely on experimental elevation of IOP, which is the major risk factor in development and progression of disease. In rats, chronic exposure to elevated IOP has been shown to lead to retinal ganglion cell death and cupping of the optic nerve, pathology which is similar to human glaucoma, thus supporting the relevance of the rat ocular hypertensive model to chronic glaucoma in humans. In our experiments we used animals with normal pressures (20-23 mmHg) to test the efficacy of LTHC to decrease IOP. It should be noted that the IOP-lowering effect of cannabinoids in our study was evident, but not as high as reported by other investigators (Pate et al., 1996; Song and Slowely, 2000). It is likely that a greater decrease in IOP may be obtained with ocular hypertensive and glaucomatous animals. Also, it is possible that the difference in the degree of IOP-
lowering effect of cannabinoids seen in our experiments, as compared to other studies, may reflect different drug delivery methods, different doses used, different IOP measurement techniques employed, as well as differences in species used. Differences in anatomy and/or physiology among different animals may account for the different effects reported with various pharmacological agents, including cannabinoid agonists. For example, most of the studies that investigated the IOP-lowering effects of cannabinoid agonists, administered these drugs by the topical route (Chien et al., 2003; Navesh et al., 2000; Mikawa et al., 1997). With respect to differences in AH outflow among species, AH outflow in rat and rabbit occurs mainly through the TM, with the uveoscleral drainage negligible (Bill, 1986, 1966). In monkey eyes, 45-70% of the AH is drained through TM, with the remainder leaving the eye through the uveoscleral pathway (Bill, 1971). Finally, in human eyes the AH outflow occurs mostly through the TM, with only 5-20% of AH leaving the eye through the uveoscleral route (Bill and Phillips, 1971). These differences may affect drug distribution and bioavailability in ocular tissues. In addition, the expression of various receptors on individual cell types involved in AH inflow or outflow may contribute to species variation in IOP regulation.

4.4 Mechanisms of Cannabinoid Action

The actions of cannabinoids on IOP, while previously have been thought to be mediated via the CNS, are now known to occur primarily through local interactions (Colasanti et al 1984; 1986; for review see, Järvinen et al., 2002). The distribution of cannabinoid receptors in different eye tissues suggests that endogenous cannabinoids may be involved in the regulation of IOP by acting at multiple ocular sites (Straiker et al.,
1999). In the human eye, immunoreactivity for CB1Rs has been identified in the CBE, trabecular meshwork and Schlemm’s canal (Straiker et al., 1999; Porcella et al., 1998). In the rat, CB1Rs have been reported to be present in the CBE and TM. Although CB2Rs have been identified in both human and rat retina (Lu et al., 2000), these receptors are absent in ocular structures known to be involved in the modulation of IOP. Also, the topical application of synthetic CB2R agonist JWH-133 has been shown to have no effect on IOP in rabbit (Laine et al., 2003).

Our study using Brown Norway rats, demonstrated that mRNA for CB1Rs was present in ciliary processes and that strong immunoreactivity for CB1Rs was found on NPCE cells, as well as positive immunostaining in the CB muscle fibres and the TM. In addition, CB1Rs were identified in NPCE and HTM5 cell lines; this includes both monomeric as well as oligomeric forms of CB1Rs. The functional relevance of oligomerized forms of CB1R was not investigated in this study. It is possible that the receptor oligomerization may be important for receptor trafficking to the cell surface and/or ligand binding or receptor internalization. For example, the GABA$_{B(1)}$ receptor isoform, when expressed alone, is retained intracellularly as immature glycoprotein (Couve et al., 1998). On the other hand, the GABA$_{B(2)}$ receptor isoform, when expressed alone, is transported to the cell surface but does not bind GABA or promote intracellular signalling (White et al., 1998). These studies indicated that both, GABA$_{B(1)}$ and GABA$_{B(2)}$ are mandatory for the functional receptor, a situation which may also be relevant for other receptor subtypes such as CB1Rs.

The presence of prominent staining for CB1Rs in the CBE cells corroborates previous findings and further suggests that the stimulation of these receptors with
cannabinoid compounds may alter AH secretion. Consistent with this, WIN55,212-2 has been reported to decrease aqueous humor inflow in rabbits by >60%, while outflow was unchanged (Sugrue et al., 1996). Similarly in primates, a single dose or multiple doses of WIN55,212-2 in normal and glaucomatous monkey eyes was reported to decrease IOP in part by decreasing aqueous flow with little alteration in outflow facility (Chien et al., 2003). The mechanism by which cannabinoid agonists decrease aqueous humor inflow is not clear, but the ability of the CB1R antagonist SR141716A to eliminate the IOP-lowering effects of cannabinoids suggests that this action is mediated via CB1Rs and may involve alterations in the sympathetic tone of the CB (Sugrue, 1997; Porcella et al., 2001; for review see, Järvinen et al., 2002).

The hypotensive action of cannabinoids in the eye may also involve vasodilatory effect on blood vessels, followed by a decrease in capillary pressure within the CB, a decrease in AH formation and a consequent decrease in IOP (Randall and Kendall, 1998; Hodges et al., 1997). Furthermore, high immunoreactivity for CB1Rs is also found in CM, an ocular structure, which alters the accommodation of the lens and allows focusing on distant objects, suggesting a role for cannabinoids in that structure. A recent study showed that application of the endocannabinoid, anandamide, or the CB1R agonist, CP55,940 to bovine CM strips produced a concentration-dependent contractile response (Lograno and Romano, 2004), an effect which was abolished by the treatment of strips with the CB1R antagonist, SR141716A. The contraction of CM, which is attached to TM, alters TM shape and may modulate IOP. Another mechanism that may modulate outflow facility for AH is the effect of cannabinoid agonists on pupil diameter. Number of reports showed enlargement in pupil size in human subjects following marijuana smoke.
An increase in pupil diameter is known to reduce AH outflow due to the blockade of AH flow through the conventional pathway through the TM and Schlemm’s canal. Furthermore, pharmacological agents, including hydroxyamphetamine, that increase pupil diameter have been correlated to reduction in AH outflow and increase IOP (Okada et al., 2001). Finally, the expression of CB1Rs in the TM, which lie on the AH outflow pathway suggests that the IOP-lowering effect of cannabinoid agonists may partially be regulated at this site.

Experiments in this thesis investigated if the pupil size in rabbit is regulated by LTHC and therefore is the potential site in regulation of AH outflow by cannabinoid agonists. We showed that topical administration of LTHC does not have an effect on pupillary diameter, and most likely does not modulate AH flow. Also, in this thesis we investigated regulation of contractility of TM cells by synthetic cannabinoid agonist, WIN55,212-2, and compared the action of WIN55,212-2 to other agents, which are known to modulate TM and IOP. The contractility of TM cells is modulated by alterations in MLC phosphorylation and this phosphorylation is crucial for CSK reorganization in both smooth muscle cells and non-muscle cells (Honjo et al., 2002). Studies have shown that the contraction of the TM cells with agents such as H-7 or ML-9 decreases AH outflow and increases IOP (Sabany et al., 2000; Honjo et al., 2002). On the other hand, the relaxation of TM cells resulted in an increase in AH outflow and decreases in IOP (Rao et al., 2001; Fukiage et al., 2001; Honjo et al., 2002). In bovine TM, the muscarinic receptor agonist, carbachol, increased contraction (Thieme et al., 2001). Our experiments are consistent with this finding. More specifically, we showed that the treatment of HTM5 cells with carbachol induces changes in cell morphology,
which include alterations in actin CSK with the development of focal adhesion and stress fibres. This action most likely results from phosphorylation of MLC, which have been shown not only to contract the cells but also to induce stress fibres and focal adhesions (Wettschureck and Offermanns, 2002; Totsukawa et al., 2000). When the effects of WIN55,212-2 were examined, we showed that a 5 minute treatment of HTM5 cells with WIN55,212-2 increases MLC phosphorylation in a dose-dependent manner, an effect which was blocked by pre-treatment of cells with selective CB1R antagonist/inverse agonist SR141716A.

MLC phosphorylation in many cells involves both Ca^{2+}-dependent and Ca^{2+}-independent pathways (Somlyo and Somlyo, 1994; Hartshorne et al., 1998). The treatment of HTM5 cells with WIN55,212-2 (10 μM) results in a rise in intracellular Ca^{2+}, an effect which is blocked by SR141716A, or by incubation of cells in Ca^{2+} free solution (Bass et al., 2005). Interestingly, our study also showed that a 15 minute treatment of cells with WIN55,212-2 decreases pMLC. The decreases in pMLC after longer period of drug exposure may be due to desensitization of CB1Rs (Sim-Selly, 2003). It is possible that cannabinoid agonists produce biphasic effects on IOP with an initial contraction of TM cells, followed by their relaxation. The phalloidin staining of HTM5 cells treated with WIN55,212-2 seems to support this hypothesis. The most noticeable changes in the first few minutes of treatment with WIN55,212-2 was an alteration in TM cell shape and reorganization of actin filaments, effects which are consistent with increased contractility of TM cells. However, after 10 minutes of treatment with the WIN55,212-2, rounding of cell bodies and development of cytoplasmic projections occurred. No focal adhesion and actin stress fibres, which are the
hallmarks of tissue contractions (Totsukawa et al., 2000; Wettschereck and Offermanns, 2002), were observed with more chronic treatment of the cells with WIN55,212-2. The observed changes in morphology of HTM5 cells upon treatment with this drug suggests that the WIN55,212-2 causes an initial increase in contractility of TM cells, followed by relaxation. The OMTC analysis further confirms the initial contraction of the HTM5 cells, which is indicated by stiffening of cells after 90 sec treatment with WIN55,212-2. Whether longer exposure of cells to WIN55,212-2 will differentially affect CSK stiffness is currently under investigation.

At this point, it should be pointed out that the initial contractility of TM cells treated with cannabinoid agonist would be expected to increase IOP in in vivo animal models. However the initial increase in IOP upon exposure to cannabinoid agonists has not been reported by any other investigators, possibly due to the fact that this effect occur rapidly and may be masked by a decrease in AH production by CBE.

4.5 Conclusion

Our experiments showed that LTHC significantly reduces IOP in rats, and provide sufficient levels of free Δ9-THC in ocular tissues to produce measurable pharmacodynamic effects. The liposomal preparation of Δ9-THC provides an effective route of delivery that may be used not only in the treatment of ocular diseases, but also in the management of pain, motor disorders and drug addiction, supporting development of other liposome-encapsulated cannabinoid compounds, including those with non-psychoactive properties.
We also showed that cannabinoids act at TM cells, which line the outflow pathway for AH. WIN55,212-2 produced biphasic effects, with initial contraction of TM cells, followed by their relaxation. The relaxation of TM cells by cannabinoids may increase AH outflow and consequently decrease IOP. Although other ocular tissues are involved in the modulation IOP, a better understanding of TM cell physiology and the ocular endocannabinoid system is essential for development of new treatments for glaucoma that are targeted at AH outflow.
CHAPTER 5

Future Studies
Future studies will further investigate the role of CB1Rs in the regulation of AH dynamic and explore hypothesis that cannabinoid agonists produce IOP-lowering effects by either decreasing AH production and/or by increasing the AH outflow through both conventional and unconventional pathways. Future avenues for investigation may address the following questions:

The modulation of TM by cannabinoid agonists alters the dynamic of AH in eye. We have shown that the exposure of TM cells to the synthetic cannabinoid agonist WIN55,212-2 causes biphasic effect in these cells, with initial changes in their morphology consistent with contraction, followed by their relaxation. Do these changes in cell morphology translate into changes in AH dynamic and consequent reduction in IOP? Experiments designed to measure the dynamics of AH in response to cannabinoid application will further our understanding.

Detailed description of molecular mechanisms that underlie cannabinoid mediated changes in TM morphology will lead to a better understanding of the physiology of AH regulation and identified novel targets for pharmacological interventions in related pathologies. To explore the signalling pathways, which are activated upon binding of cannabinoid agonist, different pharmacological agents may be used to identify the specific components of pathways hypothesised to be involved in CSK changes in TM cells. For example, if the TM contractility upon CB1R activation is mediated through the protein kinase C, we would be able to diminish this effect by using protein kinase inhibitor.

The presence of CB1R oligomeric complexes in TM cells, demonstrated through the immunocytochemical staining, allows us to speculate that CB1R oligomerization may
play a role in the cannabinoid response. Established roles for receptor oligomerization in the receptor trafficking to the cell surface, binding of the agonists to the receptor or receptor internalization may be hypothesized. Future, studies may manipulate the sequence of CB1R, causing their mutation(s) at the site where the receptors attach to each other and with an aid of functional assays may provide explanation for the physiological role in CB1R oligomerization.

We have shown that the TM response to cannabinoids is short lasting. Future studies will investigate the brevity of this response. The possibility of down-regulation of CB1Rs upon acute and chronic treatment of animals with cannabinoid agonists can be evaluated with Western Blot analysis and RT-PCR technique.

The IOP-lowering effect of LTHC observed in this study was transient, but possibly could be prolonged by modification of the liposomes, providing a more sustain release of Δ9-THC. Our results have shown that liposomal encapsulation, as a delivery system for cannabinoid compounds, may hold promise for the treatment of ocular diseases. The incorporation of non-psychoactive cannabinoids, such as cannabidiol, into liposomes may provide not only efficient delivery system for these compounds but also should minimize the potential side effects and could be tested for their ability to decrease both IOP and retinal neuroprotection in animal models.

Finally, it is possible that some of the effects of cannabinoid agonists might be mediated by yet undiscovered cannabinoid receptors or receptor subtypes. The availability of new cannabinoid receptor antagonists, with their action more specific at CB1Rs should clarify whether the IOP-lowering effect produced by cannabinoid agonists is exclusively due to the activation of CB1Rs, CB1R subtype, or a 'new' cannabinoid
receptor. The use of transgenic animals, such as CB1R-knockout mice, can also be employed to investigate if part of IOP-lowering effect of cannabinoid agonists is mediated by a 'new' cannabinoid receptor.
References


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