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THE EFFECTS OF CANNABIDIOL ON THE ELECTRICAL AND CONTRACTILE PROPERTIES OF CARDIOMYOCYTES

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THE EFFECTS OF CANNABIDIOL ON THE ELECTRICAL AND CONTRACTILE PROPERTIES OF CARDIOMYOCYTES

Ramez Ali Mansour

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Medical Sciences

Under the Supervision of Dr. Murat Oz

October 2014
Declaration of Original Work

I, Ramez Ali Mansour, the undersigned, a graduate student at the United Arab Emirates University (UAEU) and the author of this thesis entitled “The effects of cannabidiol on the electrical and contractile properties of cardiomyocytes”, hereby solemnly declare that this thesis is an original research work that has been done and prepared by me under the supervision of Dr. Murat Oz, in the College of Medicine and Health Sciences at the UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or similar title at this or any other university. The materials borrowed from other sources and included in my dissertation have been properly cited and acknowledged.

Student’s Signature____________________ Date__________________
Approval of the Master Thesis

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Department of …
College of …
Signature__________________________ Date__________________

2) Member:
Title:
Department of …
College of …
Signature__________________________ Date__________________

3) Member:
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Department of …
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Signature__________________________ Date__________________

4) Member (External Examiner):
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Institution:
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This Master thesis is accepted by:

Dean of the College of Medicine and Health Science: Professor Dennis Templeton
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Dean of the College of Graduate Studies: Professor Nagi Wakim
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Copy ____ of ___
Abstract

In earlier studies, cannabidiol (CBD), a major nonpsychotropic cannabinoid found in cannabis plant, has been shown to influence cardiovascular functions under various physiological and pathological conditions. In the present study, the effects of CBD on contractility and electrical properties of rat ventricular myocytes were investigated. Video edge detection was used to measure myocyte shortening. Intracellular Ca$^{2+}$ was measured in cells loaded with the fluorescent indicator fura-2 AM. CBD (1 µM) caused a significant decrease in the amplitudes of electrically-evoked myocyte shortening and Ca$^{2+}$ transients. However, the amplitudes of caffeine-evoked Ca$^{2+}$ transients and the rate of recovery of electrically-evoked Ca$^{2+}$ transients following caffeine application were not altered. Whole-cell patch-clamp technique was employed to investigate the effect of CBD on the characteristics of action potentials (APs) and L-type Ca$^{2+}$ channels. CBD (1 µM) significantly decreased the duration of APs. Further studies on L-type Ca$^{2+}$ channels indicated that CBD inhibits these channels with IC50 of 0.1 µM in a voltage-independent manner.

Keywords: Cannabidiol, I type calcium channels, ventricular myocyte.
Title and Abstract in Arabic

آثار الكانابيديول على الخواص الكهربية و الانقباضية الخاصة بالخلايا العضلية القلبية

الملخص

في دراسات سابقة، الكانابيديول، وهو من المؤثرات العقلية الكانابينويدية الرئيسية الموجودة في نبات الحشيش، وجد أن له تأثيرات على وظائف القلب والأوعية الدموية تحت مختلف الظروف الفيسيولوجية والمرضية.

الهدف في هذه الأطروحة هو دراسة أثر الكانابيديول على الانقباضات و الخصائص الكهربية لخلايا القلب العضلية و تم قياس انقباض الخلايا العضلية بالاستعانة بطريقة الكشف بالفيديو إيدج. و تم قياس Ca²⁺ الموجود داخل الخلايا باستخدام مؤشر فلوريسنت (fura-2 AM).

أهم نتائج هذه الدراسة هو أن الكانابيديول (1 μM) تسبب في انخفاض ملحوظ في سعة انقباض الخلايا العضلية المثارة كهربياً و معدل Ca²⁺ transients (Ca²⁺ العابر) غير أن ال Ca²⁺ العابر المثير بالكافيين و معدل Ca²⁺ الإشعاع ال Ca²⁺ العابر كهربياً التالية لعملية إضافة الكافيين لم تتغير. و تم التحقق من تأثير الكانابيديول على خصائص جهد الفعل (action potentials) و قنوات ال L-type Ca²⁺ بالاستعانة بتقنية الالتقاط الرقعي لكامل الخلية (Whole-cell patch-clamp). بالإضافة إلى ذلك، دلت النتائج أن الكانابيديول (1 μM) أدى لملاحظة انخفاض ملفوفظ في المدة الزمنية لجهد الفعل. مزيد من الدراسات على قنوات ال L-type Ca²⁺ بينت أن الكانابيديول يمكن أن يمنع ملاحظة اللهجة بالمقدار IC50 من 0.1 μM (voltage-independent manner).
Acknowledgments

I would like to express my deep gratitude to my master thesis advisor, Professor Murat Oz who has supported me throughout my thesis with his patience and knowledge whilst allowing me the room to work in my own way. I appreciate his encouragement and efforts without which this thesis would not have been completed. One simply could not wish for a better or friendlier supervisor. I am also grateful to Professor Chris Howarth, Professor Oleg Krishtal and Dr. Rajesh Mohanraj for spending time in reading and providing useful suggestions about the thesis.

My gratitude also goes to my colleagues to thank them for their assistance to me: Mr. Anwar, Dr. Nour Alain, Dr. Lina and Khawla. Additionally, I want to acknowledge some friends who inspired my efforts to overcome difficulties, so a special thanks to, Mohammed and Abrar.

I am grateful to my mother, my father and my uncle for all of the sacrifices that they have made on my behalf. I would not have got to this point without you. Special thanks go to my brother Ahmed and my sister Yousra for their continuous encouragement.
Dedication

To my parents, my uncle, my brother Ahmed and my 
sister Yousra
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## Glossary

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>THC</td>
<td>Tetrahydrocannabinol</td>
</tr>
<tr>
<td>CBN</td>
<td>Cannabinol</td>
</tr>
<tr>
<td>CBD</td>
<td>Cannabidiol</td>
</tr>
<tr>
<td>THCA</td>
<td>Tetrahydrocannabinolic Acid</td>
</tr>
<tr>
<td>CBGA</td>
<td>Cannabigerolic Acid</td>
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<td>Cannabigerol</td>
</tr>
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<tr>
<td>CBCA</td>
<td>Cannabichromenic Acid</td>
</tr>
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<td>Cannabichromene</td>
</tr>
<tr>
<td>AEA</td>
<td>Anandamide</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-Arachidonylglycerol</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty Acid Amidehydrolase</td>
</tr>
<tr>
<td>MAGL</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>AJA</td>
<td>Ajalemic Acid</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient Vanilloid Acceptor 1</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome Proliferator Activated Receptor</td>
</tr>
<tr>
<td>VR1</td>
<td>Vanilloid Receptors</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PKC</td>
<td>Phosphokinase C</td>
</tr>
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<td>NADA</td>
<td>N-arachidonoyl-Dopamine</td>
</tr>
<tr>
<td>NCX</td>
<td>Soduim- Calcium Exchanger</td>
</tr>
<tr>
<td>APD</td>
<td>Action Potential Duration</td>
</tr>
<tr>
<td>RyRs</td>
<td>Ryanodine Receptors</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<tr>
<td>APD</td>
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Chapter 1: Introduction

1.1 Aims and Objectives:
The aims of this research are to examine the therapeutic potential and mechanism by which the phytocannabinoid Cannabidiol CBD affects cardiac contractility. This will be achieved by investigating the effects of CBD on action potential duration, ventricular shortening, myofilament sensitivity, and L-type calcium current.

1.2 History
Marijuana is a crude preparation of flowering tops, leaves, seeds and stems from female Indian hemp cannabis sativa plants (Figure 1). It has been used for centuries for both recreational and therapeutic purposes. Currently cannabis is being investigated for the treatment of various pathological conditions such as pain, nausea, vomiting and cancer. It is one of the most extensively studied plants. There were more than 12,000 PubMed articles published in January 2014 using the key word cannabis in relation to the various biological and pharmacological effects of this drug.
Figure 1. Cannabis Sativa Leaves. ScienceDaily, n.d. Web. 30 Apr. 2014

Figure 2. Major Phytocannabinoids
The medicinal use of marijuana has a long history of therapeutic use, going back several thousands of years. It was often used for the same medical conditions it is used to treat today such as muscle spasms, pain, nausea, vomiting, epilepsy, and glaucoma. However, use of marijuana is also associated with distorted perception (sights, sounds, time and touch), panic attacks, trouble with thinking, difficulties in learning and problem solving, and loss of motor coordination. Due to these adverse effects, the use of marijuana was banned in the U.S.A and throughout most of the world almost eighty years ago.

During the second half of the 20th century, researchers discovered that the biological actions of marijuana are mediated mainly by a group of chemicals collectively called cannabinoids. Although cannabis plant extracts contain more than 460 compounds, based on pharmacological properties and radioligand binding studies, currently about 60 of the compounds found in this plant, are collectively named phytocannabinoids. Among these phytocannabinoids, Δ⁹-Tetrahydrocannabinol (THC) is a major chemical that mediates the psychoactive actions of the cannabis plant (Figure 2). In addition to THC, other cannabinoids such as Cannabigerol, Cannabichromene, Cannabinol (CBN) and Cannabidiol (CBD), constitute major non-psychoactive components of the cannabis plant. These compounds are highly lipophilic and bear significant similarities in their chemical structure. For example, CBD which is the focus of this study, is a structural isomer of THC i.e. it has the same chemical composition but the atoms are arranged differently.
While the majority of the adverse psychological effects of marijuana are known to be mediated by THC, the contribution of other cannabinoids such as CBD and CBN to the overall influence of marijuana remains unknown. In recent years, various combinations of these psychoactive and non-psychoactive cannabinoids have been used in cannabis based treatments. Different extracts of cannabis have been used therapeutically to relieve, asthma, pain, whooping cough, epilepsy and inflammation. However, due to the psychoactive effects of cannabis such as anxiety, cognitive impairment and hallucinations, medicinal use of these compounds was discontinued (Robson, 2001).

Nevertheless, its therapeutic potential to solve a myriad of medical problems has attracted considerable research interest. Thus, in recent years research has focused on the therapeutic potential of the plant to develop new chemicals that retain the therapeutic benefits without causing psychotropic effects.
Figure 3. Illustrating Major Phytocannabinoids Synthetic Pathways.
1.3 Cannabinoids

Despite its use for centuries, the active ingredients and chemical constituents of the cannabis plant remained largely unknown until the beginning of the 1960s when the main psychoactive constituent, THC was extracted and identified (Mechoulam & Gaoni, 1965). In the 1980s, other compounds with chemical structures similar to that of THC were synthesized by various drug companies in an attempt to discover new drugs to alleviate pain. Some of these newly synthesized compounds such as CP55,940, CP55,244, HU-210, JWH-018 displaced radioactively labeled THC in radioligand binding experiments and lead to the discovery of the first cannabinoid receptor (CB1) in the brain (Herkenham et al., 1990). In the 1990’s CB1 and CB2 receptors were cloned, expressed functionally and identified pharmacologically (Pertwee, 1997).

Further studies in this field, indicated that, similar to opioids, there are endogenous ligands that can bind and activate cannabinoid receptors. These findings eventually lead to the discovery of “endocannabinoids” i.e. endogenously produced cannabinoids. Endocannabinoids mainly N-arachidonylethanolamine (Anandamide; AEA) and 2-arachidonylglycerol (2-AG) have considerable agonistic effects on the cannabinoid receptors (Mechoulam et al., 1995), and mimic most of the pharmacological actions of THC.
1.3.1 Phytocannabinoids

As mentioned earlier the most abundant constituents of the cannabis plant are THC and CBD. Within the plant, phytocannabinoids are synthesized from fatty acid precursors via a series of transferase and synthase enzymes (Figure 3). The two major phytocannabinoids, THC and CBD, are derived from a common synthetic precursor, cannabigerol. From a pharmaco-chemical perspective, whilst THC and CBD have pentyl side chains, major homologues are Δ9-tetrahydrocannabivarin and cannabidivarin, respectively, with propyl side chains, derived from cannabigerovarin. Importantly, despite only small differences in chemical structure, these compounds appear to exhibit markedly different pharmacological properties (Hill, Williams, Whalley, & Stephens, 2012).

THC has a wide spectrum of pharmacological effects ranging from anti-inflammatory and analgesic actions to cancer treatment, mainly through the activation of the cannabinoid receptors (Mechoulam, Parker, & Gallily, 2002). However, CBD has no agonistic activity on cannabinoid receptors, but it is capable of producing various pharmacological effects by acting on diverse groups of target proteins ranging from enzymes and receptors to neurotransmitter transporters and ion channels (Hill et al., 2012).
1.3.2 Endocannabinoids

Endocannabinoids

Endogenous ligands for the cannabinoid receptor are synthesized from polyunsaturated fatty acids and fatty acid derivatives from glycerol. They mimic the actions of phytocannabinoids by binding to cannabinoid receptors (Di Marzo, 2008). In addition to AEA and 2-AG, other endocannabinoids with similar fatty acid-based chemical structures have also been identified in the last decade (Pertwee et al., 2010). Some of these endocannabinoids include 2-arachidonylglycerol ether (Noladin ether); (Hanus et al., 2001), NADA, and virodhamine (Pertwee et al., 2010; Porter et al., 2002).

Endocannabinoids, unlike neurotransmitters, are neither synthesized nor stored in the neurons. Therefore they are not released by a synaptic process, but produced in response to cellular activity (Di Marzo et al., 1994). It has been shown that biological processes such as membrane depolarization and activation of metabotropic receptors cause increases in intracellular Ca²⁺ levels and activate enzymes involved in the synthesis of endocannabinoids (Di Marzo, 2008). Following their synthesis, AEA and 2-AG are degraded by enzymatic hydrolysis by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (Cravatt & Lichtman, 2002).

Endocannabinoids can also be transported out of the cell by diffusion through cell membranes or by a mechanism that involves an unidentified transporting protein located in the plasma membrane (Cravatt & Lichtman, 2002; Glaser, Kaczocha, and Deutsch, 2005).
1.3.3 Synthetic Cannabinoids

Following the discovery of cannabinoid receptors there have been attempts to produce compounds with therapeutic effects such as anti-inflammatory/analgesic and anti-emetic effects without inducing the psychotropic effects. As a result various chemicals such as CP55,940, CP55,244,HU-210, JWH-018, and JWH-073 have been synthesized (Huffman et al., 1998). These compounds are known collectively as synthetic cannabinoids (Ashton, 2012). Currently, little is known about the biological activities of these compounds with their diverse chemical structures. However, recent reports indicate that various combinations of synthetic cannabinoids are employed for recreational purposes throughout the world in order to avoid legal regulations related to phytocannabinoids (Burstein and Zurier, 2009). However, the synthetic cannabinoid Nabilone has recently been approved by the FDA (U.S Food and Drug Administration) and recommended for the treatment of chemotherapy-induced nausea and vomiting that is unresponsive to conventional antiemetics (Ware, Daeninck, and Maida, 2008). In addition nabilone is used for the treatment of anorexia and weight loss in AIDS patients (Burstein and Zurier, 2009; Pertwee et al., 2010). Nabilone has also been reported to be an effective pain killer for patients with fibromyalgia (Skrabek, Galimova, Ethans, & Perry, 2008).

Currently the following cannabinoid based medications have been approved by the FDA and are commercially available (Schicho & Storr, 2011): 1– Sativex® (GW Pharmaceuticals), for pain and spasticity in patients with multiple sclerosis. Currently, four different formulations of Sativex® are under investigation, including the high THC extract (Tetranabinex®), THC:CBD (narrow ratio), THC:CBD (broad ratio) and the high CBD extract (Nabidiolex®) (Scuderi et al. 2009). Currently, three
Sativex® delivery systems exist: the oromucosal spray, sublingual tablets and inhaled dosage forms. In 2005, the oromucosal spray administration of Sativex® was approved for the treatment of multiple sclerosis symptoms (Perras 2005). To date, Sativex preparations have been licensed in Canada, the UK and Spain. 2– Marinol® (Solvay Pharmaceuticals, Belgium), oral capsules containing dronabinol (a synthetic THC), are recommended as an appetite stimulant and antiemetic. 3– Cesamet® (Valeant Pharmaceuticals International), is an oral capsule containing nabilone (a synthetic THC analog), for patients with chemotherapy-induced nausea and vomiting. This drug is currently licensed in Canada, the USA and the UK.
<table>
<thead>
<tr>
<th>Phytocannabinoids</th>
<th>Name</th>
<th>Action of CB Receptors</th>
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<tbody>
<tr>
<td>Δ9 Tetrahydrocannabinol (Δ9 THC)</td>
<td>Non selective CB1/CB2 agonist</td>
<td></td>
</tr>
<tr>
<td>Cannabigerol (CBG)</td>
<td>CB1 selective antagonist</td>
<td></td>
</tr>
<tr>
<td>Cannabidiol (CBD)</td>
<td>CB1 antagonist</td>
<td></td>
</tr>
<tr>
<td>Cannabichromene (CBC)</td>
<td>No action on CBRs</td>
<td></td>
</tr>
<tr>
<td>Cannabinol (CBN)</td>
<td>Non selective CB1/CB2 agonist</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Endocannabinoids</th>
<th>Name</th>
<th>Action of CB Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Arachidonylethanol Amide (Anandamide)</td>
<td>Non selective CB1/CB2 agonist</td>
<td></td>
</tr>
<tr>
<td>2-Arachidonylglycerol (2-AG)</td>
<td>Non selective CB1/CB2 agonist</td>
<td></td>
</tr>
<tr>
<td>O-Arachidonylethanolamine (Virodamine)</td>
<td>Non selective CB1/CB2 agonist</td>
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<tr>
<td>Synthetic Cannabinoids</td>
<td>CP55,940</td>
<td>Non selective CB1/CB2 agonist</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td>WIN55,212</td>
<td>Non selective CB1/CB2 agonist</td>
</tr>
<tr>
<td></td>
<td>Sch.336</td>
<td>CB2 selective</td>
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<tr>
<td></td>
<td>Ajulemic acid (AJA)</td>
<td>Non selective CB1/CB2 agonist</td>
</tr>
</tbody>
</table>
1.4 Cannabidiol

CBD is the main non-psychoactive component of cannabis and constitutes up to 40% of cannabis extracts (Grlic, 1962). It was first isolated in 1940 and its chemical structure was identified in 1963 (Mechoulam and Hanus, 2002). In recent years, CBD has been considered to be a promising therapeutic agent for clinical use, since, in addition to its non-psychotropic properties, it exhibits as well as low toxicity in humans and has a high therapeutic index and a low teratogenic potential (Rosenkrantz, Fleischman, and Grant, 1981; Campos, Moreira, Gomes, Del Bel, and Guimaraes, 2012; Scuderi et al., 2009). An orally-administered liquid containing CBD has received orphan drug status in the US, for use as a treatment for Dravet Syndrome, a rare-form of epilepsy that is resistant to treatment with current anti-epileptic drugs (Gloss, Nolan, and Staba, 2014).

Cannabidiol is insoluble in water but soluble in organic solvents, such as DMSO and alcohol. It is rapidly distributed when administered intravenously, easily passes the blood-brain barrier and has a prolonged elimination from the body (Samara, Bialer and Harvey, 1990).

1.4.1 Mechanisms of Action of Cannabidiol

In general, CBD has been shown to act on a wide range of molecular and cellular target sites. Some of these molecular targets are various types of membrane receptors such as GPR55 (Ryberg et al., 2007), opioid (Kathmann, Flau, Redmer, Trankle and Schlicker, 2006) and 5HT1A receptors as well as Transient Vanilloid receptors 1 (TRPV1) (Bisogno et al., 2001), and Peroxisome Proliferator Activated Receptor (PPARγ) (Bishop-Bailey, 2000). Significantly, CBD, at low µM concentrations acts as an antagonist for CB1 and CB2 receptors (Pertwee, Ross, Craib and Thomas, 2002). Other
proteins that CBD interacts with include voltage-gated and ligand-gated ion channels, and various types of enzymes (for review see Pertwee, 2008; Izzo et al., 2009; Campos et al., 2012; Garcia-Arencibia et al., 2007). These cellular and molecular targets are discussed in detail in the following sections.

**a. Actions of cannabidiol on the cannabinoid system:**

In earlier clinical studies, CBD, with no detectable activity by itself, was reported to attenuate some of the effects of THC such as anxiety and tachycardia (Karniol, Shirakawa, Kasinski, Pfeferman and Carlini, 1974). Similarly, it was found that the pharmacological actions of cannabinoid receptor agonists can be antagonized by low (nM range) concentrations of CBD in a mouse’s vas deferens (Pertwee et al., 2002). These effects of CBD are unlikely to involve direct interaction at cannabinoid receptor CB1 and CB2 binding sites except at high concentrations of CBD, since CBD appears to have a relatively low affinity for cannabinoid receptors. Ki values for CBD-induced displacement of a radiolabelled ligand from cannabinoid CB1 and CB2 receptor binding sites have been reported to be 4.35 and 2.86 µM, respectively in one study (Showalter, Compton, Martin and Abood, 1996) and >10 µM in other experiments (Devane, Dysarz, Johnson, Melvin and Howlett, 1988; Bisogno et al., 2001). Further studies indicated that CBD behaves as a high-potency antagonist for cannabinoid receptor agonists in a mouse’s brain tissue and in membranes from CHO cells transfected with cannabinoid receptors (Thomas et al., 2007). It was also reported that the function of a novel receptor GPR55 is inhibited by low concentrations of CBD (Ryberg et al., 2007).
The interaction of CBD with the cannabinoid system is not limited to cannabinoid receptors. Tissue levels of endogenously produced cannabinoids are also affected by CBD. For example, it has been shown that CBD inhibits the uptake and the enzymatic hydrolysis of AEA (Bisogno et al., 2001). Furthermore, CBD has been shown to cause activation of cannabinoid receptors indirectly by enhancing tissue concentrations of endocannabinoids (Fowler, 2004). In functional experiments it has been suggested that some of the effects of CBD sensitive to CB receptor antagonists are mediated by an entourage effect of CBD on tissue levels of endocannabinoids (Fowler, 2004).

**b. Effects of cannabidiol on other membrane receptors:**

CBD has been shown to stimulate Vanilloid Receptors (VR1) with an efficacy comparable to that of capsaicin, the natural agonist of this receptor (Bisogno et al., 2001). The stimulation of the VR1 receptor by capsaicin and some of its analogues leads to fast desensitization, with subsequent analgesic and anti-inflammatory effects. It has been suggested that, similar to capsaicin, CBD also causes desensitization of VR1 and enhances the effect of capsaicin on VR1, suggesting that CBD exerts its anti-inflammatory actions, in part, by desensitization of sensory nociceptors (Bisogno et al., 2001). CBD has been reported to act as a weak agonist (EC$_{50}$ = 3.5 µM) on human TRPV1 receptors in HEK293 cells expressing these receptors (De Petrocellis et al., 2011). CBD has also been demonstrated to act as an agonist on TRPV2 (Qin et al., 2008) and TRPA1 receptors, while it acts as an antagonist on the TRPM8 receptor (De Petrocellis et al., 2008).

It was also shown that at a µM concentration range, CBD displaces the 8-OH-DPAT, 5-HT1A receptor agonist, from cloned human 5-HT1A receptors expressed in
cultured cells obtained from a Chinese hamster ovary (E. B. Russo, Burnett, Hall and Parker, 2005). In subsequent studies, results of several in vivo experiments have supported the involvement of 5-HT1A receptors in the effect of CBD (Campos et al., 2012). At high µM concentrations (10-100 µM), CBD has also been shown to interact with other G-protein coupled receptors such as 5HT2 and opioid receptors in an allosteric manner (E. B. Russo et al., 2005; Kathmann, et al., 2006).

**c. Effect of cannabidiol on second messengers:**

Calcium is considered one of the most important second messengers involved in the regulation of various cell functions, such as muscle contraction and the release of neurotransmitters. It has been shown that CBD treatment increases intracellular concentrations of Ca$^{2+}$ in cultured hippocampus neurons (Drysdale, Ryan, Pertwee and Platt, 2006; Ryan, Drysdale, Lafourcade, Pertwee and Platt, 2009). In another study, the anti-epileptic activity of CBD has been suggested to be due to its bidirectional regulatory role on intracellular Ca$^{2+}$ levels (Ryan et al., 2009). In another investigation, it was proposed that CBD increases intracellular Ca$^{2+}$ levels under normal physiological conditions, but decreases Ca$^{2+}$ levels under highly excitable conditions (Mato, Victoria Sanchez-Gomez and Matute, 2010; Rao and Kaminski, 2006). This action of CBD was believed to be due to the regulation of Ca$^{2+}$ transport in mitochondria which acts as a sink for intracellular Ca$^{2+}$ (Ryan et al., 2009).

In addition to its influence on intracellular stores, CBD also acts on active Ca$^{2+}$ transport proteins such as Ca$^{2+}$-ATPase in myocytes (Gilbert, Pertwee and Wyllie, 1977) and neurons (Gilbert et al., 1977; Ryan et al., 2009). The highly lipophilic nature of CBD grants it easy access to intracellular targets such as cellular organelles (endoplasmic
reticulum and mitochondria) and intracellularly located enzymes that contribute to CBD-induced alterations in intracellular Ca$^{2+}$ levels (Collins and Haavik, 1979). In this context, it is important to note that, the functions of various Ca$^{2+}$-activated enzymes such as phospholipase C (PLC) (Howlett, Scott and Wilken, 1989), phospholipaseA2, lipoxygenase (Takeda, Usami, Yamamoto and Watanabe, 2009) and Phosphokinase C (PKC) (Hillard and Auchampach, 1994; White and Tansik, 1980) are also modulated by low µM concentrations of CBD.

In addition to Ca$^{2+}$, CBD acts on the uptake process of several neurotransmitters (for a review see Pertwee 2008). CBD decreases the uptake of $[^3]$H adenosine in both murine microglia and macrophages, and binding studies show that CBD binds to the nucleoside transporter (Carrier, Auchampach and Hillard, 2006). The enhancement of adenosine signaling through inhibition of its uptake was suggested as providing non-cannabinoid receptor mechanism by which CBD can decrease inflammation (Carrier et al., 2006).

d. Cannabidiol effects on voltage-gated ion channels:

Earlier studies of motor neurons showed that CBD depresses the amplitude of action potential suggesting that it inhibits the function of neuronal voltage-gated Na$^+$ channels (Turkanis and Karler, 1986). However, there has been no further study investigating the effect of CBD on Na$^+$ channels. Another study indicated that CBD increased the L-type Ca$^{2+}$ current recorded in the hippocampal neurons (Drysdale et al., 2006). Similarly to these Na$^+$ channels, there have been no further studies investigating the actions of CBD on L-type Ca$^{2+}$ channels. On the other hand, the effect of CBD on T-type voltage-gated Ca$^{2+}$ channels has been investigated in recent years. It has been shown
that CBD inhibits different subtypes of CaV3 family channels (Ross, Napier and Connor, 2008).

e. **Cannabinoid effects on ligand-gated ion channels:**

In HEK-293 cells expressing glycine receptors, CBD showed a positive allosteric modulatory effect in a low µM concentration range (Ahrens et al., 2009). The EC50 values for the potentiating effects of CBD were 12.3 µM for α1 and 18.1 µM for α1β1 subunit combinations. Direct activation of glycine receptors was also observed at CBD concentrations above 100 µM. Similarly, glycine-mediated currents recorded in dorsal horn neurons of rat spinal cord slices were potentiated by CBD applications (Xiong et al., 2012). Further investigations indicated that mutations of the α1 subunit TM2 serine residue to isoleucine abolished the co-activation and the direct activation, of the glycine receptor by CBD (Foadi et al., 2010). In in vivo experiments, it has been shown that systemic and intrathecal administration of CBD significantly suppressed chronic inflammatory and neuropathic pain without causing apparent analgesic tolerance in rodents (Xiong et al., 2012). The analgesic potency of CBD and 11, similarly structured cannabinoids, is positively correlated with potentiation of the α3-subunit glycine receptor. In contrast, analgesia induced by these cannabinoids is neither correlated with their binding affinity for CB1 and CB2 receptors nor with their psychoactive side effects. Furthermore, NMR analysis revealed a direct interaction between CBD and S296 in the third transmembrane domain of purified α3 GlyR. More importantly, the CBD-induced analgesic effect was absent in mice lacking the α3 subunit from the glycine receptor suggesting that the α3 subunit mediates glycinerigeric CBD-induced suppression of chronic pain (Xiong et al., 2012).
Another ligand-gated ion channel reported to be modulated by CBD is the α7-nicotinic cholinergic receptor (Mahgoub et al., 2013). It was shown that the function of human α7-nicotinic receptors expressed in xenopus oocytes was inhibited by CBD with an IC50 value of 11.3 µM in a non-competitive manner. Significantly, the 5-HT3 receptor, with structural similarities to the α7-nicotinic receptor was also shown to be inhibited in xenopus oocytes and HEK-293 cells in an allosteric manner (K. H. Yang et al., 2010).
1.4.2 Clinical Applications

a. Anti-inflammatory actions

As the most abundant non-psychoactive cannabinoid in the plant, the anti-inflammatory actions of CBD and its analogs have been studied extensively in recent years (Burstein and Zurier, 2009; Booz, 2011). CBD reduces joint inflammation in collagen-induced arthritis in mice (Sumariwalla et al., 2004) and carrageenan paw edema in rats (Costa et al., 2004). In addition, oral administration of CBD (2.5–20 mg/kg) reduces neuropathic and inflammatory pain in rats. This effect is reversed by vanilloid but not by CB receptor antagonists (Costa, Trovato, Comelli, Giagnoni and Colleoni, 2007). CBD also reduces intestinal inflammation in mice (Capasso et al., 2008). In addition to its ability to suppress production of the inflammatory cytokine TNFα, CBD appears to exert anti-inflammatory pressure by suppressing the fatty acid amidohydrolase (FAAH), thereby increasing concentrations of the anti-inflammatory endocannabinoid anandamide. Further, insight into mechanisms whereby CBD exerts therapeutic effects was provided by experiments which indicated that CBD attenuates inflammation induced by high glucose levels in diabetic mice (Rajesh et al., 2007). Specifically, CBD treatment reduces mitochondrial superoxide, inducible nitric oxide synthase, nuclear factor kappa β activation, and transendothelial migration of monocytes (Burstein and Zurier, 2009; Booz, 2011). Another potential therapeutic use of CBD may lie in its ability to counter some undesirable effects of THC (sedation, psychotropic effects, tachycardia), thus suggesting that if given together with THC, it may allow higher doses of THC (E. Russo & Guy, 2006). THC and CBD have been administered as an oral mucosal spray to 58 patients with rheumatoid arthritis (Blake, Robson, Ho, Jubb and McCabe, 2006). Treated
patients had a significant reduction in pain and an improvement in sleep compared to patients given a placebo.

b. Neuroprotection

In earlier studies, CBD has been shown to normalize glutamate homeostasis (Hampson et al., 2000; El-Remessy et al., 2003), reduce oxidative stress (Hampson, Grimaldi, Axelrod and Wink, 1998; Marsicano, Moosmann, Hermann, Lutz, and Behl, 2002), attenuate glial activation and the occurrence of local inflammatory events (Ruiz-Valdepenas et al., 2011; Martin-Moreno et al., 2011). It appears that there may be two key mechanisms underlying the neuroprotective effects of CBD. The first is the capability of CBD to restore the normal balance between oxidative events and antioxidant endogenous mechanisms (Fernandez-Ruiz, 2012) that are frequently disrupted in neurodegenerative disorders, thereby enhancing neuronal survival. The second is CBD as a neuroprotective compound, and its anti-inflammatory activity. The anti-inflammatory effects of CBD are related to the control of microglial cell migration and the toxicity exerted by these cells, i.e. the production of pro-inflammatory mediators (Fernandez-Ruiz, 2012).

The neuroprotective effects of CBD were also evaluated in animal models with Parkinson’s disease, Huntington’s disease and neonatal ischemia. Pathological changes induced by 6-hydroxydopamine, a toxic compound that targets catecholaminergic cells, were significantly reduced by CBD treatment (Lastres-Becker, Molina-Holgado, Ramos, Mechoulam and Fernandez-Ruiz, 2005; Mechoulam, et al., 2002). It appears that these reductions after CBD treatment were irreversible, as they did not recover when CBD was halted (Mechoulam, Peters, Murillo-Rodriguez and Hanus, 2007). CBD was also useful
in preventing beta-amyloid–induced neurodegeneration in in vivo and in vitro models of Alzheimer disease (Fernandez-Ruiz, 2012). Increased production of reactive oxygen species, lipid peroxidation, DNA fragmentation, and intracellular $\text{Ca}^{2+}$ concentrations induced by beta-amyloid peptide were significantly reduced after CBD treatment of PC12 cells (Iuvone et al., 2004).

CBD has also been shown to be protective against neuronal damage due to ischemia (Hampson et al., 2000). In rats subjected to middle cerebral artery occlusion, infarct size and neurological impairment were reduced by 50-60% by CBD. Similarly, post-ischemic administration of CBD protected against hyperlocomotion and neuronal injury following middle cerebral artery occlusion in gerbils (Braida et al., 2003). Furthermore, it was recently shown that CBD treatment reduced the infarct volume in brain ischemia, and that this effect was independent of cannabinoid receptor type 1 and transient receptor potential V1, but sensitive to the 5-HT1A receptor antagonist, WAY100135 (Hayakawa et al., 2004; Mishima et al., 2005) suggesting that activation of 5-HT1A receptors mediate the neuroprotective effects of CBD.

c. **Treatment of nausea and vomiting**

Cannabis has long been used to treat nausea and vomiting induced by various drugs and pathological conditions (Parker, Rock and Limebeer, 2011). Several combinations of phytocannabinoids and synthetic cannabinoids have currently been approved for use in the treatment of chemotherapy-induced nausea and vomiting (Parker et al., 2011). The reduction of nausea and vomiting demonstrated by cannabis sativa use *in vivo* and *in vitro* has been attributed to the presence of THC and CBD. Unlike THC, which exerts its action via CB1 receptors, CBD mimics the anti-nausea and anti-vomiting
properties of THC through mechanisms unrelated to CB receptor activation (Mechoulam et al., 2007). Interestingly, one of the most effective antiemetic drugs used in clinics is a 5HT\textsubscript{3} receptor antagonist (Thompson, Chau, Chan and Lummis, 2006). 5HT\textsubscript{3} receptors belong to a superfamily of ligand-gated ion channels that mediate serotonin-induced rapid depolarizations in intestinal neurons and regulate peristaltic activity of intestinal smooth muscle (Izzo and Camilleri, 2009). CBD and THC have been shown to inhibit 5HT\textsubscript{3} receptors (K. H. Yang et al., 2010) and suppress emesis induced by various chemical and physical stimuli (Parker et al., 2011).
d. Cardiovascular effects

i) Anti-arrhythmic role of CBD:

Studies examining the cardioprotective effects of CBD showed that CBD has antiarrythmic effects after an ischemic reperfusion injury (Walsh, Hepburn, Kane and Wainwright, 2010). In models of myocardial infarction following ligation of the left anterior descending coronary artery, in vivo treatment with CBD resulted in a reduction in infarct size (Rajesh et al., 2010). This finding has been attributed to an immuno-modulatory effect in CBD, since it was accompanied by a reduction in leukocyte infiltration and interleukin 6 concentrations. A single dose of CBD given 10 minutes prior to ischemia or reperfusion resulted in a reduction in infarct size, and a significant reduction in the incidence of ventricular tachycardia and total number of ventricular ectopic beats (Stanley, Hind and O'Sullivan, 2013).

The CB1 receptor antagonist, AM251, also demonstrates antiarrhythmic properties, and treatment with AM251 co-administered with CBD has a synergistic effect, suggesting that activation of CB1 receptors is proarrhythmic (Walsh et al., 2010). The synergism observed, which persists when CB1 receptors are blocked prior to CBD administration, is suggestive of cross talk between CB1 and other CB receptors in the ischemic heart (Walsh et al., 2010). Furthermore, CBD significantly reduces cardiac dysfunction in mice with diabetes. CBD treatment has been shown to decrease myocardial inflammation, reduce oxidative stress as demonstrated by a reduction in nuclear factor-kB activation, suppress mitogen-activated protein kinase activation, and reduce the expression of adhesion molecules and tumor necrosis factor (Walsh et al., 2010).
ii) Vascular effects of CBDs:

CBDs exert vasodilative effects as demonstrated by in vivo and in vitro models (see Stanley, Hind and O'Sullivan, 2013). However, the mechanism and potency of CBD appear to differ between various experimental models. For example, in some studies, CBD acts through cyclooxygenase, while other experiments use through CB1 receptor antagonism and the inhibition of potassium (K⁺) channel hyperpolarization. The size of the blood vessel is also a key factor, as maximal responses to anandamide (AEA) are observed in small resistance vessels (O'Sullivan, Kendall and Randall, 2005).

In rat aorta, time-dependent CBD vasodilatation has been shown to be mediated through Gᵢₒ protein that is sensitive to pertussis toxin, but is not sensitive to CB1 antagonism or TRPV1 desensitization (O'Sullivan, Sun, Bennett, Randall and Kendall, 2009). Additional differences are observed in other species. The mechanism by which vasodilatation occurs is also dependent on the type of cannabinoid. For instance, AEA and N-arachidonoyl-dopamine (NADA) exert similar vasodilatation effects in rat aorta; however, these effects occur through different mechanisms (Stanley, et al., 2013).

It has also been demonstrated that CBD induces vasodilatation of segments of human mesenteric artery preconstricted with U46619 and endothelin-1. The authors of this study determined that mesenteric artery relaxation is endothelium-dependent, and involves CB1 receptor and TRPV channel activation, NO release and K⁺ channel hyperpolarization (Stanley et al., 2013).

iii) Relationship between CBD and peroxisome proliferator-activated receptor gamma:
Nuclear receptors are proteins found within cells that regulate diverse functions, such as homeostasis, reproduction, development, and metabolism. Peroxisome proliferator-activated receptor gamma (PPARγ) belongs to a family of nuclear receptors and plays a crucial role in glucose and lipid homeostasis, in addition to cell proliferation, cell differentiation, and inflammatory responses (Bishop-Bailey, 2000; Hsueh and Bruemmer, 2004). There is evidence to suggest that cannabinoids bind to and activate PPARγ, thereby causing PPAR-mediated responses. THC, which is the major component of cannabis, causes time-dependent endothelium-dependent and PPARγ-mediated vasodilatation of rat aorta. In contrast, CBD, which is a weak agonist of PPARγ receptors, causes increased PPARγ transcriptional activity in PPARγ-overexpressing HEK293 cells (Hsueh and Bruemmer, 2004).

Since CBD is a weak PPARγ receptor agonist, side effects normally associated with PPARγ receptor activation, such as weight gain, edema, and increased plasma lipoprotein can be avoided. Therefore, CBD may prove to have therapeutic potential as a low affinity agonist (O'Sullivan et al., 2009; Walsh et al., 2010).

iv) Hemodynamic effects of CBD:

Studies on the hemodynamic effects of CBD proved inconclusive. In some studies, a 16-mm Hg reduction in mean arterial blood pressure was noted, with no change in heart rate (Walsh et al., 2010). The authors of this study concluded that the effects of CBD are best seen in models with elevated blood pressure (Walsh et al., 2010). However, other studies did not demonstrate significant hemodynamic changes (Bergamaschi, Queiroz, Zuardi and Crippa, 2011).
CBD is reported to have anxiolytic properties, and is effective as a treatment for a fear of public speaking. CBD reduced heart rate and blood pressure responses in Wistar rats conditioned by fear. This response is believed to be mediated by 5HT$_{1A}$ receptors, since the effects were inhibited by the 5HT$_{1A}$ receptor antagonist, WAY100635 (Gomes, Resstel and Guimaraes, 2011; Zuardi, 2008).

v) Vascular protective effects of CBD:

High glucose levels are known to contribute to endothelial dysfunction in patients with diabetes. High glucose levels promote the inhibition of endothelial NO, decrease the vasodilatation effects and increase the vasoconstrictor effects of prostanoids, increase superoxide production, and increase ROS production (Vanhoutte, Shimokawa, Tang and Feletou, 2009). The aforementioned effects of high glucose were reduced when cells were co-incubated with CBD (Rajesh et al., 2007). Key elements of atherosclerosis, such as monocyte adhesion and transendothelial migration are reduced by CBD. Neither CB1 nor CB2 receptors appear to be responsible for mediating these effects of CBD (Rajesh et al., 2007). Treatment with CBD may also protect against diabetic retinopathy. CBD prevented vascular hyperpermeability at the blood-retinal barrier (BRB), and protected the retina against oxidative damage, inflammation, and increased levels of cell adhesion molecules in an in vivo model of diabetic retinopathy (El-Remessy et al., 2006).
1.5 Contraction of Cardiac Muscle

Contraction of cardiac muscle cells or cardiomyocytes shows certain characteristics specific to this muscle. For example, contraction of cardiomyocytes is not initiated by neurons as in skeletal muscle but by electrical excitation originating from the heart’s own pacemaker, the sinoatrial node, which generates spontaneous and periodic action potentials. When an action potential is initiated in one cell, current flows through the gap junctions and depolarizes neighboring cells. If depolarization causes the membrane potential to be more positive than the threshold, self-propagating action potentials occur in the neighboring cells as well. Thus, the generation of an action potential is just as critical for initiating contraction in cardiac muscle as it is in skeletal muscle, but it is triggered by the sinoatrial node and the specialized conduction system of the heart (Figure 4).

![Electrical System of the Heart](image)

**Figure 4. Anatomy and Function of the Heart's Electrical System. Johns Hopkins Medicine, Baltimore, Maryland.**
Separate tubular structures, the transverse tubules (T tubules), cross the cell. In the cardiac myocyte, the T tubule crossings occur at the Z-line, in contrast to the A-I junction in skeletal muscle. The lumen of the T tubule is continuous with the extracellular fluid surrounding the cell and, as in skeletal muscles, the action potential is propagated down the T tubule (Ferrantini et al., 2013). Adjacent cardiac myocytes are joined end-to-end at structures known as intercalated disks. These always occur at a Z-line (Figure 5). At these points, the cell membranes form a number of parallel folds and are tightly held together by desmosomes. This results in strong cell-to-cell cohesion, thus allowing the contraction of one myocyte to be transmitted axially to the next. Gap junctions exist between cardiac muscle cells, providing low resistance pathways for the spread of excitation from one cell to another.

Figure 5. Adjacent cardiac myocytes are joined end-to-end at structures known as intercalated disks.
Figure 6. Ventricular action potential membrane currents that generate a normal action potential. Resting (4), upstroke (0), early repolarization (1), plateau (2) and final repolarization are the 5 phases of the action potential. A decline of potential at the end of phase 3 in pacemaker cells, such as the sinus node, is shown as a broken line. The inward currents, INa, ICa and If, are shown in yellow boxes; the sodium-calcium exchanger (NCX) is also shown in yellow. It is electrogenic and may generate inward or outward current. IKach, IK1, Ito, IKur, IKr, and IKs are shown in gray boxes. The action potential duration (APD) is approximately 200 ms (Nattel & Carlsson, 2006).
1.5.1 Excitation–Contraction Coupling

a. The ventricular action potential

The cardiac action potential represents changes in the membrane potential due to the movement of ions across the cell membrane through voltage-gated ion channels, pumps, and exchangers, and has distinct characteristics in different regions of the heart. In ventricular cardiomyocytes (Amin, Tan and Wilde, 2010). According to kinetic properties, the cardiac action potential is divided into 4 distinct phases (Figure 6). The first phase of the action potential is characterized by the activation of fast Na\(^+\) channels, which open briefly to produce an influx of positive Na\(^+\) ions causing rapid depolarization of the cell membrane and an upstroke of the action potential. This initial phase is called phase 0. Following phase 0, a brief repolarization is observed at the peak of the action potential and is the consequence of both fast Na\(^+\) channel inactivation and the activation of initial repolarizing currents. This brief repolarization phase is called phase 1.

Following phase 1, a long lasting plateau of depolarization (100-200 ms), named phase 2, is observed. Depolarizing currents, mainly late Na\(^+\) and L-type Ca\(^{2+}\) channels stay open and continue to offset repolarizing currents during the plateau phase. Finally, L-type Ca\(^{2+}\) channels begin to inactivate and several cardiac K\(^+\) channels such as delayed-rectifier and inward-rectifier K\(^+\) channels begin to open and cause the repolarization of the membrane to resting membrane potential. This repolarization is called phase 3. The ventricular action potential is regenerative, and continues to excite ventricular cells if the stimulus exceeds the critical threshold for depolarization as determined by availability of Na\(^+\) current. Phase 4 is the resting membrane potential, and describes the membrane potential when the cardiomyocyte is not being stimulated. In a great majority of contracting
cardiomyocytes phase 4 has a low slope (almost a horizontal line). However, in pacemaking cells, phase 4 is unstable (phase 4 - is the pacemaker potential). In pacemaker cells (such as the Sinoatrial node), the membrane slowly depolarizes during phase 4, until it reaches a threshold potential (around -40mV) or until it is depolarized by an electrical impulse coming from another cell. The reason for this pacemaker potential is an increased inward current of sodium (Na\(^+\)) through voltage-dependent channels, but also an increased inward Ca\(^{2+}\) current and a decrease in the K\(^+\) outward current. These Na\(^+\) channels, in cardiac pacemaker cells, contrary to what usually happens in other cells, open when the voltage is more negative.

b. Calcium-induced calcium release

The action potential propagates along the sarcolemma, invades the transverse tubules and causes release of Ca\(^{2+}\) from internal Ca\(^{2+}\) stores such as sarcoplasmic reticulum and leads eventually to the initiation of muscle contraction. This sequence of events, beginning with action potential generation and resulting in muscle contraction is known as excitation-contraction coupling (Figure 7). Initially, depolarization caused by the action potential activates voltage-gated L-type Ca\(^{2+}\) channels which are concentrated in the Tubules (Shepherd and McDonough, 1998). Activation of these channels induces a conformational change in the ion channel structure and causes a subsequent influx of Ca\(^{2+}\) into the cell. This influx of Ca\(^{2+}\) is known as sparklets (Navedo and Santana, 2013). The presence of sparklets is not sufficient to cause activation of the contractile machinery. However, it is sufficient to initiate the release of further Ca\(^{2+}\) from intracellular stores by interacting with ryanodine receptors (RyRs) on the sarcoplasmic reticulum. The local amplification of intracellular Ca\(^{2+}\) mediated by RyR activation is also known as a
Ca$^{2+}$ spark (Fearnley, Roderick and Bootman, 2011), and is essential for causing a Ca$^{2+}$ transient that activates the contractile myofilaments resulting in muscle contraction. In general, cytosolic Ca$^{2+}$ influx in ventricular cells occurs mainly through two distinct sources. About 8 to 23% of Ca$^{2+}$ enters extracellularly through the opening of L-type Ca$^{2+}$ channels and 77% to 92% of total Ca$^{2+}$ employed in muscle contraction is released from the sarcoplasmic reticulum through RyRs, although some inter-species differences do exist (Bers, 2008). The contribution of NCX and mitochondria to cytosolic Ca$^{2+}$ is usually not more than 1% (Bers, 2000). Following its release, Ca$^{2+}$ is pumped back into the sarcoplasmic reticulum by a Ca$^{2+}$-ATPase pump and also removed from the cell via the Na$^+/Ca^{2+}$ exchanger located at the plasma membrane (Wang, Song, Lakatta and Cheng, 2001). In cardiac muscle, the activity of a Ca$^{2+}$-ATPase in the sarcoplasmic reticulum is inhibited by the regulatory protein phospholamban. When phospholamban is phosphorylated by cAMP-dependent protein kinase, its ability to inhibit the Ca$^{2+}$-ATPase is lost. Thus, activators of cAMP-dependent protein kinase, such as the neurotransmitter epinephrine, may enhance the rate of cardiac myocyte relaxation.
Calcium Channels

Due to the importance of Ca\(^{2+}\) in various vital cellular events such as muscle contraction, neurotransmitter release, and exocytosis; the regulation of Ca\(^{2+}\) entrance through voltage-gated Ca\(^{2+}\) channels has been studied in detail (Catterall, Perez-Reyes, Snutch and Striessnig, 2005). According to pharmacological and biophysical characteristics three different types of Ca\(^{2+}\) channels have been characterized. These different types of Ca\(^{2+}\) channels are summarized in Table 2.
In the heart, both T-type and L-type Ca$^{2+}$ channels have been shown to be expressed (Zhou and January, 1998). However, T-type Ca$^{2+}$ channels appear to be localized mainly in cells involved in heart rhythm and automaticity such as pacemaker and atrial cells, while L-type Ca$^{2+}$ channels are localized mainly in ventricular myocytes. As mentioned above, due to their slow activation kinetics, L-type Ca$^{2+}$ channels require long depolarizations lasting 100-200 ms. After the activation process, L-type Ca$^{2+}$ channels are inactivated by various factors which include time, voltage, cytosolic Ca$^{2+}$ concentration and calmodulin (Bers, 2000).
Table 2. Classification of calcium channels

<table>
<thead>
<tr>
<th>Ca(^{2+}) current type</th>
<th>(\alpha_1) subunits</th>
<th>Specific blocker</th>
<th>Principal physiological functions</th>
<th>Inherited diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>Ca(_{v}) 1.1</td>
<td>DHPs</td>
<td>Excitation-contraction coupling in skeletal muscle, regulation of transcription</td>
<td>Hypokalemic periodic paralysis</td>
</tr>
<tr>
<td></td>
<td>Ca(_{v}) 1.2</td>
<td>DHPs</td>
<td>Excitation contraction coupling in cardiac and smooth muscle, endocrine secretion, neuronal Ca(^{2+}) transients in cell bodies and dendrites, regulation of enzyme activity, regulation of transcription</td>
<td>Timothy syndrome: cardiac arrhythmia with developmental abnormalities and autism spectrum disorders</td>
</tr>
<tr>
<td></td>
<td>Ca(_{v}) 1.3</td>
<td>DHPs</td>
<td>Visual transduction</td>
<td>Stationary night blindness</td>
</tr>
<tr>
<td>N</td>
<td>Ca(_{v}) 2.1</td>
<td>(\omega)-CTx-GVIA</td>
<td>Neurotransmitter release, Dendritic Ca(^{2+}) transients</td>
<td>Familial hemiplegic migraine, cerebellar ataxia</td>
</tr>
<tr>
<td>P/Q</td>
<td>Ca(_{v}) 2.2</td>
<td>(\omega)-Agatoxin</td>
<td>Neurotransmitter release, Dendritic Ca(^{2+}) transients</td>
<td>Familial hemiplegic migraine, cerebellar ataxia</td>
</tr>
<tr>
<td>R</td>
<td>Ca(_{v}) 2.3</td>
<td>SNX-482</td>
<td>Neurotransmitter release, Dendritic Ca(^{2+}) transients</td>
<td>Familial hemiplegic migraine, cerebellar ataxia</td>
</tr>
<tr>
<td>T</td>
<td>Ca(_{v}) 3.1</td>
<td>None</td>
<td>Pacemaking and repetitive firing</td>
<td>Absence seizures</td>
</tr>
<tr>
<td></td>
<td>Ca(_{v}) 3.2</td>
<td>None</td>
<td>Pacemaking and repetitive firing</td>
<td>Absence seizures</td>
</tr>
<tr>
<td></td>
<td>Ca(_{v}) 3.3</td>
<td>None</td>
<td>Pacemaking and repetitive firing</td>
<td>Absence seizures</td>
</tr>
</tbody>
</table>

Abbreviations: DHP, dihydropyridine; \(\omega\)-CTx-GVIA, \(\omega\)–conotoxin GVIA from the cone snail Conus geographus; SNX-482, a synthetic version of a peptide toxin from the tarantula Hysterocrates gigas (Catterall, 2011).

As shown in Figure 8, the L-type Ca\(^{2+}\) channel consists of 4 subunits: the major pore-forming \(\alpha_1\) subunit and a variety of accessory subunits. The \(\alpha_1\) subunit: Formed of 4 homologous domains (I–IV), each consisting of 6 transmembrane (TM) helices joined by intracellular links. The loops between TM5 and TM6 form the channel pore, while TM4 contains arginine or lysine residues that form the voltage sensor, which is thought to move or twist as the membrane potential changes.

Ion selectivity is governed by a glutamate residue preserved in the pore-lining loop of all 4 domains (Dolphin, 2006; J. Yang, Ellinor, Sather, Zhang and Tsien, 1993). Two Ca\(^{2+}\) ions enter the cell at a time, are regulated by 2 glutamate residues and flow down the Ca\(^{2+}\) concentration gradient (Heinemann, Terlau, Stuhmer, Imoto and Numa, 1992; J. Yang et al., 1993). Lanthanum, cobalt and cadmium inhibit the \(\alpha_1\) subunit
(Dolphin, 2009). Expression of the $\alpha_1$ subunit without the auxiliary subunits, results in low L-type Ca$^{2+}$ channel expression levels, and abnormal voltage and kinetic properties in the channel. The $\beta$ subunit is located on the intracellular side of the channel. It interacts with the $\alpha$ subunit through the $\alpha$-interaction domain (AID), a guanylate cyclase–like region located between domains I and II (Catterall, 2011; Pragnell et al., 1994). The AID has a well-conserved gene sequence and also occurs in N-type and P/Q-type Ca$^{2+}$ channels (Richards, Butcher and Dolphin, 2004). The $\beta$ subunit alters channel expression, voltage dependence, and gating kinetics. Four $\beta$ subunits have been cloned, and all except for $\beta_{2a}$ hyperpolarize activation and steady-state inactivation of Ca$^{2+}$ (Dolphin, 2003). The $\alpha_{2\delta}$ subunit is an accessory subunit consisting of the $\delta$ transmembrane domain, and $\alpha_2$, an extracellular domain. Four $\alpha_2$ isoforms have been identified. Co-expression of $\alpha_{2\delta}$ subunits increases Ca$^{2+}$ channel expression and function. The $\gamma$ subunit is a 30 to 40kD inhibitory protein consisting of 4 TM$\alpha$ helices (Takahashi, Seagar, Jones, Reber and Catterall, 1987).
Secretion of catecholamines from the adrenal medulla and the autonomic nervous system stimulates β-adrenergic receptors via adenylyl cyclase, cAMP, and protein kinase A (PKA) to produce positive chronotropic, inotropic and lusitropic effects. Cardiac L-type Ca\(^{2+}\) channels are modulated through the β-adrenergic/cAMP signaling pathway; \(\alpha_1\) and β subunits are phosphorylated by PKA (De Jongh et al., 1996; Haase, Bartel, Karczewski, Morano & Krause, 1996; Hell et al., 1993; Puri, Gerhardstein, Zhao, Ladner and Hosey, 1997). β-adrenergic receptor activation causes a 2-fold increase in the L-type Ca\(^{2+}\) current by increasing the number of channels and their activation probability. The latter is mediated through PKA-mediated phosphorylation of the Ca\(_{\text{v}1.2}\) (\(\alpha_{1C}\)-subunit) channel protein and/or associated proteins (Catterall, 2010). As opposed to the adrenergic system, reciprocal control over Ca\(^{2+}\) entry is provided by ACh, acting through muscarinic Ach
receptors, raising intracellular cGMP concentrations and causing cGMP-dependent phosphorylation of the L-type Ca\(^{2+}\) channels. In turn, the cGMP-dependent phosphorylation of L-type Ca\(^{2+}\) channels, at sites distinct from those phosphorylated by the cAMP-dependent kinase, causes a decrease in Ca\(^{2+}\) influx during the cardiac action potential and thus a decrease in the force of contraction.
1.5.2 Sarcoplasmic Reticulum and Ryanodine Receptors

The sarcoplasmic reticulum has many roles in the regulation of muscle contraction and intracellular Ca\(^{2+}\) homeostasis. The release of Ca\(^{2+}\) from this organelle causes increased Ca\(^{2+}\) levels to activate various Ca\(^{2+}\) dependent enzymes such as calmodulin and adenyl cyclase (Fearnley et al., 2011). It is also the site of phospholipid generation and it communicates with other intracellular organelles, such as the mitochondria. However, it’s most important function is the regulation of Ca\(^{2+}\) homeostasis. It is well known that cytosolic Ca\(^{2+}\) levels during resting conditions are in the nM range, while extracellular Ca\(^{2+}\) concentrations are much higher (μM range). As mentioned above, following an action potential, cytosolic Ca\(^{2+}\) concentration increases and activates ryanodine receptors, which amplify the Ca\(^{2+}\) signal and stimulate Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Several isoforms of ryanodine receptors (RyR) have been identified: RyR1 is expressed in skeletal muscle, RyR2 in cardiac muscle and the brain, and RyR3 in the brain (Lanner, Georgiou, Joshi and Hamilton, 2010). Although the ryanodine receptor is a protein of the SR membrane, 80% of this protein is located in the cytoplasm. The intracellular position of ryanodine differs from one cell type to another (Zucchi and Ronca-Testoni, 1997). It can be directly attached (as in skeletal muscles) or detached (as in cardiac muscles) to the L-type Ca\(^{2+}\) channel. In skeletal muscle, direct interaction of the ryanodine receptor with the L-type Ca\(^{2+}\) channel causes fast excitation-contraction coupling and rapid initiation of skeletal muscle contraction (within a few minutes). In cardiac muscle on the other hand, excitation-contraction is a relatively slow process (within hundreds of minutes). Function of the ryanodine receptor is also regulated by several ligands, including Ca\(^{2+}\), calmodulin and caffeine (Fearnley et al., 2011).
1.5.3 The Contractile Machinery

Myofilaments occupy about half of the cell volume in mammalian ventricular myocytes. As with skeletal muscle, cardiac myocytes contain the contractile proteins actin (thin filaments) and myosin (thick filaments) together with the regulatory proteins troponin and tropomyosin. In their structural configuration, the thin filament comprises actin with a spiraling backbone of tropomyosin, along with troponin complexes (C, I and T) that occur at certain intervals (Figure 9). Each actin filament consists of a site where myosin can bind, but these sites are blocked by tropomyosin when cytosolic Ca\(^{2+}\) concentration is low. When cytosolic Ca\(^{2+}\) concentration increases due to Ca\(^{2+}\) release, Ca\(^{2+}\) binds to troponin C causing tropomyosin to shift, thereby exposing the myosin binding sites. Adenosine diphosphate (ADP) and phosphate, present on the myosin head since the previous movement cycle, are released when myosin attaches to myosin binding sites on the actin filaments. The energy generated in the head is used to initiate upstroke and sliding, until the actin–myosin complex is liberated by adenosine triphosphate (ATP) binding the myosin head. ATP is hydrolyzed to ADP and phosphate, releasing energy that is stored in the myosin head to be used in the subsequent stroke.
Figure 9. Sarcomere structure. The thin filament is composed of actin, tropomyosin, and the troponin which consists of the troponins Tn-T, Tn-C, and Tn-I. The thick filament is composed of myosin, a globular head portion (S1), a hinged stalk region (S2) and a rod section. S1 consists of an ATP hydrolysis domain and the actin binding domain, it is associated with the light-chain 1 and 2 (LC-1; LC-2) (Hamdani et al., 2008).
1.5.4 Calcium Sensitivity of Myofilaments

The term myofilament Ca\(^{2+}\) sensitivity came into use after it was discovered that the force of contraction is not only dependent on the free cytosolic Ca\(^{2+}\) concentration, but also on the affinity of troponin C for Ca\(^{2+}\). Biochemical and physiological experiments have shown that several factors affect myofilament Ca\(^{2+}\) sensitivity, including pH, temperature, ionic strength and troponin I phosphorylation (Ruegg, 2003). Furthermore, these factors can be categorized by whether they affect thick or thin filaments (Bers, 2001). Factors that affect thick filaments tend to affect the relationship between intracellular Ca\(^{2+}\) concentration and the extent of myosin light chain phosphorylation. Whereas factors that affect thin filaments, affect phosphorylation of myosin light chain and force generation due to contractile proteins (Bers, 2001).
Chapter 2: Methods

2.1 Ventricular Myocyte Isolation

Ventricular myocytes were isolated from adult male Wistar rats (264 ± 19 g) according to previously described techniques. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments at the United Arab Emirates University. In brief, the animals were euthanized using a guillotine and their hearts were removed rapidly and mounted for retrograde perfusion according to the Langendorff method (Figure 10). The hearts were perfused at a constant flow of 8 ml g heart\(^{-1}\) min\(^{-1}\) and at 36–37 °C with a solution containing (mM): 130 NaCl, 5.4 KCl, 1.4 MgCl\(_2\), 0.75 CaCl\(_2\), 0.4 NaH\(_2\)PO\(_4\), 5 HEPES, 10 glucose, 20 taurine and 10 creatine set to pH 7.3 with NaOH. When the heart had stabilized perfusion was continued for 4 minutes with a Ca\(^{2+}\)-free isolation solution containing 0.1 mM EGTA, and then for 6 minutes with cell isolation solution containing 0.05 mM Ca\(^{2+}\), 0.75 mg/ml collagenase (type 1; Worthington Biochemical Corp., USA) and 0.075 mg/ml protease (type XIV; Sigma, Germany). Ventricles were excised from the heart, minced and gently shaken in collagenase-containing isolation solution supplemented with 1% BSA. Cells were filtered from this solution at 4 minutes intervals and resuspended in an isolation solution containing 0.75 mM Ca\(^{2+}\).
Figure 10. Langendorff Heart

- Solution Chamber
- Heating Coil
- Water jacketed sugar chamber
- Peristaltic pump
2.2 Measurement of Ventricular Myocyte Shortening

Ventricular myocytes were allowed to settle on the glass bottom of a Perspex chamber mounted on the stage of an inverted microscope (Axiovert 35, Zeiss, Germany). Myocytes were superfused (3–5 ml/min) with normal Tyrode (NT) containing (mM): 130 NaCl, 5 KCl, 1 MgCl₂, 10 glucose, 5 HEPES, 20 taurine, 10 creatine and 0.75 CaCl₂ (pH 7.4). Shortening of myocytes was recorded using a video edge detection system (VED-114, Crystal Biotech, USA) according to previously described techniques (Howarth, Qureshi and White, 2002). Resting cell length (RCL) time to peak (TPK), time to half (THALF) relaxation and amplitude of shortening (expressed as a percentage of resting cell length) were measured in electrically stimulated (1 Hz) myocytes maintained at 35–36 °C. Data was acquired and analyzed with signal averager software v 6.37 (Cambridge Electronic Design, UK). Experimental solutions were prepared from stock immediately prior to each experiment.

2.3 Measurement of Intracellular Ca²⁺ Concentration

Myocytes were loaded with the fluorescent indicator fura-2 AM (F-1221, Molecular Probes, USA) as described (Howarth et al., 2002). In brief, 6.25 μl of a 1 mM stock solution of fura-2 AM (dissolved in dimethyl sulphoxide) was added to 2.5 ml of cells to give a final fura-2 concentration of 2.5 μM. Myocytes were gently shaken for 10 minutes at 24 °C (room temperature). After loading, myocytes were centrifuged, washed with NT to remove extracellular fura-2 and then left for 30 min to ensure complete hydrolysis of the intracellular ester. Intracellular Ca²⁺ was measured according to previously described techniques (Howarth et al., 2011). Myocytes were alternately illuminated by 340 and 380 nm light using a monochromator (Cairn Research, UK)
which changed the excitation light every 2 minutes. The resulting fluorescence emitted at 510 nm was recorded by a photomultiplier tube and the ratio of the fluorescence emitted at the two excitation wavelengths (340/380 ratio) was calculated to provide an index of intracellular Ca\(^{2+}\) concentration. Resting fura-2 ratio, time to peak (TPK), time to half (THALF) decay of the Ca\(^{2+}\) transient, and the amplitude of the Ca\(^{2+}\) transient were measured in electrically stimulated (1 Hz) myocytes maintained at 35-36 °C. Data were acquired and analyzed with signal averager software v 6.37 Cambridge Electronic Design, UK.

### 2.4 Measurement of Sarcoplasmic Reticulum Ca\(^{2+}\) Content
Sarcoplasmic reticulum (SR) Ca\(^{2+}\) release was assessed using previously described techniques (Howarth et al., 2002). After establishing a steady state of Ca\(^{2+}\) transients in electrically stimulated (1 Hz) myocytes maintained at 35–36 °C and loaded with fura-2, stimulation was paused for a period of 5 seconds (Figure 11). Caffeine (20 mM) was then applied for 10 seconds using a solution switching device customized for rapid solution exchange. Electrical stimulation was resumed and the Ca\(^{2+}\) transients were allowed to recover to a steady state. SR-releasable Ca\(^{2+}\) was assessed by measuring the area under the curve of the caffeine-evoked Ca\(^{2+}\) transient. Fractional release of SR Ca\(^{2+}\) was assessed by comparing the amplitude of the electrically evoked steady state Ca\(^{2+}\) transients with that of the caffeine-evoked Ca\(^{2+}\) transient and refilling of the SR was assessed by measuring the rate of recovery of electrically evoked Ca\(^{2+}\) transients following the application of caffeine.
Assessment of Myofilament Sensitivity to Ca$^{2+}$

In some cells shortening and fura-2 ratio were recorded simultaneously.

Myofilament sensitivity to Ca$^{2+}$ was assessed from phase-plane diagrams of fura-2 ratio versus cell length by measuring the gradient of the fura-2-cell length trajectory during late relaxation of the twitch contraction. The position of the trajectory reflects the relative myofilament response to Ca$^{2+}$ and hence, can be used as a measure of myofilament sensitivity to Ca$^{2+}$ (Spurgeon et al., 1992; Howarth et al., 2002).
2.6 Whole-Cell Recordings

Whole-cell recordings were performed in isolated ventricular myocytes. The myocytes were placed in the recording chamber (0.4 mL) mounted on the stage of an inverted microscope (CK2, Olympus, Tokyo, Japan) (Figure 12). After settling to the bottom of the chamber, cells were superfused with an external solution for 10 min at a rate of 2–3 mL min\(^{-1}\) at room temperature. The external solution contained (in mM) 120 NaCl, 1 EGTA, 10 MgCl\(_2\), 10 Glucose, 10 HEPES, 1 NaH\(_2\)PO\(_4\) and 5 KCl. Transmembrane currents were recorded with an axopatch amplifier 200 B (Axon Instruments, Inc., Foster City, CA, USA). The whole-cell bath solution contained (in mM): 95 NaCl, 50 TEACl, 2 MgCl\(_2\), 2 CaCl\(_2\), 10 HEPES and 10 Glucose (adjusted to pH 7.35 with NaOH). Glass microelectrodes were made using a microelectrode puller (Model P-97, Sutter Instrument Co., Novato, CA, USA) (Figure 13) by two-stage pulling, and had a resistance of 2.0–4.0 MΩ when filled with electrode internal solution containing (in mM) 140 CsCl, 10 TEACl, 2 MgCl\(_2\), 2 HEPES 1 MgATP and 10 EGTA (adjusted to pH 7.25 with CsOH). Only rod shaped cells with clear cross-striations were used for the experiments. Figure 14 shows typical ventricular myocytes with patch pipette in solution. After gigaseal formation (1–10 GΩ) by suction through a pipette, the membrane was ruptured with a gentle suction to obtain the whole-cell voltage clamp configuration. Membrane capacitance and series resistance were compensated after membrane rupture to minimize the duration of capacitive current. The experiment was conducted under room Temperature (22–24 °C). To record L-type Ca\(^{2+}\) currents, the external solution was changed to the Na\(^+\)-free solution in which Na\(^+\) was replaced by equimolar TEA (TEA–Cl). The Na\(^+\) current was also inactivated at the holding potential -50 mV. K\(^+\) currents were suppressed by substituting K\(^+\) by Cs\(^+\). Computer-generated
voltage pulses were programmed using the pCLAMP 10.0 software (Axon Instruments).

Cells with stable calcium currents were then tested using different drugs

Figure 12. Patch clamp Setup
Figure 13. Microelectrode puller (model P-97, Sutter Instrument)
Figure 14. Glass electrode forming giga seal with ventricular myocyte

Figure 15. Ventricular myocyte
Chapter 3: Results

3.1 Effects of Cannabidiol on Ventricular Myocyte Shortening

In Normal Tyrode (NT) solution, amplitudes of myocyte shortening in response to electrical stimulation (1 Hz) gradually decreased to 85-80% of controls during experiments lasting up to 20 minutes. No further running down of the shortening amplitude was observed in the NT solution containing 0.02% DMSO. And, unless it stated otherwise, DMSO (in the concentration used in CBD containing solution) was also included routinely in the control NT solutions during the shortening and Ca\(^{2+}\) transient experiments.

In initial control experiments and in line with earlier studies conducted under similar conditions it has been shown that 0.01% DMSO did not significantly affect contractile parameters and Ca\(^{2+}\) current in rat ventricular myocytes (Sun et al., 2010). The application of control solution (NT) containing dimethyl sulfoxide at the highest concentration used in contractility studies (0.02% v/v; used as vehicle for 1 μM CBD) caused 11-13% inhibition of the shortening (n=7-10; P<0.05 compared to a 0 time point).

In the first set of experiments, we tested the effect of CBD on the contractility of acutely isolated rat ventricular myocytes. Figure 16A shows typical records of shortening in myocytes superfused with either NT (in the absence of CBD, NT contained 0.02% DMSO in all experiments) or NT + 1 μM CBD and during washout with NT. The time frame of the effect of CBD on amplitude of shortening is shown in Figure 16B. The effect of CBD reached a steady level within 5 minutes of CBD application. Increasing the incubation time to 10 minutes did not cause any further change in the amplitude of shortening. Recovery from CBD inhibition was incomplete during the 5-10 minutes
washout time. In summary, the amplitude of shortening measured after 5 minutes bath application of CBD was significantly reduced (paired t-test; n=17; P<0.05) by up to 46.4 ± 3.1 % in the controls (Figure 16C). The effect of CBD increased in a concentration-dependent manner. The concentration-response relationship normalized to maximal CBD inhibition indicated that CBD suppresses shortening of amplitudes with an IC₅₀ of 0.6 µM (Figure 17). Among other contraction parameters measured, resting cell length (RCL), time to peak and time to half relaxation were not significantly altered (n=17; P>0.05) by 10 minutes superfusion with CBD.
Figure 16. Effects of cannabidiol on ventricular myocyte shortening. (A) Typical records of shortening in an electrically stimulated (1 Hz) ventricular myocytes superfused with either NT (containing the vehicle, 0.02% DMSO) or NT + 1 µM CBD and during washout with NT. (B) Time course of the mean amplitudes (AMP) of shortening expressed as a percentage of control values in vehicle (NT + 0.02% DMSO), and in presence of CBD (1 µM). Myocytes were maintained at 35-36 °C and superfused with CBD for 10 minutes. (C) Data shown as means ± S.E.M., n = 6-8 cells. * indicates statistically significant difference at the level of P< 0.05.
Figure 17. Concentration-inhibition relationship of cannabidiol effect on the shortening of myocytes. Concentration-response curve for the inhibitory effect of CBD on myocyte shortening. The data was normalized to maximal inhibitory effect of CBD and plotted as a function of CBD concentrations. Data are mean ± S.E.M. from n = 6-8 cells for each concentration.
3.2 Effects of Cannabidiol on Intracellular Ca\(^{2+}\)

In the second set of experiments we investigated the effects of 5 minutes bath application of 1 µM CBD on the resting intracellular Ca\(^{2+}\) levels, and on the amplitudes and kinetics of Ca\(^{2+}\) transients elicited by electrical-field stimulation. Typical records of Ca\(^{2+}\) transients in a myocytes superfused with NT (containing 0.02 % DMSO), NT + 1 µM CBD and during washout with NT are shown in Figure 18A. The effects of 1 µM CBD on resting fura-2 ratio, TPK Ca\(^{2+}\) transient, THALF decay of the Ca\(^{2+}\) transient and AMP of Ca\(^{2+}\) transients are shown in Figure 18B-E, respectively. Although, CBD has been shown to alter intracellular Ca\(^{2+}\) levels in various types of cells (see Pertwee, 2008; Pertwee, et al., 2010), application of 1 µM CBD for 5 to 10 minutes did not cause a significant alteration in resting fura-2 ratio, TPK Ca\(^{2+}\) transients, or THALF decay of the Ca\(^{2+}\) transient (paired t-test; n=11-14 cells, \(P>0.05\)). However, the AMP of the Ca\(^{2+}\) transient was significantly reduced by 1 µM CBD (0.337 ± 0.043 fura-2 ratio units) compared to 0.488 ± 0.056 fura-2 ratio units (paired t-test, n =11 cells) in the control experiments.
Figure 18. Effects of cannabidiol on amplitude and time-course of intracellular Ca^{2+} in rat ventricular myocytes. (A) Typical records of Ca^{2+} transients in an electrically stimulated (1 Hz) ventricular myocytes superfused with either NT or NT + 1 µM CBD and during washout with NT; scale bar indicates 0.1 fura-2 ratio unit (RU). Also shown resting fura-2 ratio (340/380 nm) (B), time to peak (TPK) Ca^{2+} transient (C), time to half (THALF) decay of the Ca^{2+} transient (D) and amplitude of the Ca^{2+} transient (E). Myocytes were maintained at 35-36 °C and superfused with CBD for 10 minutes. Data are shown as means ± SEM, n=11-14 cell. * indicates statistically significant difference at the level of P< 0.05.
3.3 Effect of Cannabidiol on Sarcoplasmic Reticulum Ca\textsuperscript{2+} Transport

The effect of CBD on sarcoplasmic reticulum Ca\textsuperscript{2+} transport was tested. Figure 19A shows a typical record illustrating the protocol used in these experiments. Initially, the myocyte was electrically stimulated at 1Hz. Electrical stimulation was then turned off for 5 seconds. Caffeine was then applied for 10 seconds using a rapid solution-exchanger device. Electrical stimulation was then restarted, and the recovery of intracellular Ca\textsuperscript{2+} was recorded during a 60 seconds period. The SR Ca\textsuperscript{2+} content was assessed by measuring caffeine-evoked Ca\textsuperscript{2+} release (under the caffeine-evoked Ca\textsuperscript{2+} transient) and fractional release of Ca\textsuperscript{2+} by comparing the amplitude of the electrically evoked steady-state Ca\textsuperscript{2+} transients with that of the caffeine-evoked Ca\textsuperscript{2+} transient in the presence of either NT alone or NT with 1 µM CBD. Maximal amplitudes and the rates of Ca\textsuperscript{2+} release by 20 mM caffeine remained unaltered after a 10 minute bath application of 1 µM CBD (Figure 19B) and (Figure 19C; paired t-test; n = 7 cells, \(P>0.05\)). Fractional release of SR Ca\textsuperscript{2+} was not significantly altered in 1 µM CBD compared to NT (0.78 ± 0.04 in CBD versus 0.81 ± 0.07 in controls; paired t-test; n = 8 cells; Figure 19D). The recovery of the Ca\textsuperscript{2+} transients during electrical stimulation following application of caffeine (Figure 19E) was also not significantly altered in myocytes exposed to 1 µM CBD myocytes compared to the control cells (paired t-test; n = 8 cells, \(P>0.05\)).
Figure 19. Effect of cannabidol on sarcoplasmic reticulum Ca\(^{2+}\) transport. (A) Typical records illustrating the experimental protocol. (B) Comparison of the effect of caffeine application on the area under caffeine-evoked Ca\(^{2+}\) transients in NT and CBD-treated ventricular myocytes. (C) Area under the curve. (D) Mean amplitude of SR fractional release of Ca\(^{2+}\). (E) Recovery of electrically evoked intracellular Ca\(^{2+}\) after application of caffeine. Data are mean ± S.E.M., n = 7-8 cells.
3.4 Effect of Cannabidiol on Myofilament Sensitivity to Ca$^{2+}$

The effects of CBD on myofilament sensitivity to Ca$^{2+}$ were also investigated. These experiments tested whether CBD decreases the mechanical response by altering the affinity of the contractile machinery of the ventricular myocytes to intracellular Ca$^{2+}$.

Atypical record of myocyte shortening, fura-2 ratio and phase-plane diagrams of fura-2 ratio versus cell length in myocytes exposed to NT is shown in Figure 20A. The gradient of the trajectory reflects the relative myofilament response to Ca$^{2+}$ and hence, has been used as a measure of myofilament sensitivity to Ca$^{2+}$ (Spurgeon, et al., 1992; Howarth, et al., 2002). The gradients of the fura-2-cell length trajectory during late relaxation of the twitch contraction measured during the periods 500-600 ms (Figure 20B), 500-700 ms (Figure20C), and 500–800 ms (Figure20D) were not significantly altered in CBD compared to NT suggesting that myofilament sensitivity to Ca$^{2+}$ is not reduced by CBD (CBD-treatment was compared to NT containing 0.02 % DMSO, paired t-test; n = 17 cells; $P>0.05$).
Figure 20. Effect of cannabidiol on myofilament sensitivity to Ca$^{2+}$. (A) Typical record of myocyte shortening and fura-2 ratio and phase-plane diagrams of fura-2 ratio vs. cell length in a myocyte exposed to NT. The arrow indicates the region where the gradient was measured. B-D shows the effects of 1 µM CBD on the mean gradient of the fura-2 cell length trajectory of the twitch contraction during the periods 500-600 (B), 500-700 (C) and 500-800 ms (D) of late relaxation. Data are mean ± S.E.M., n = 17 cells.
3.5 Effects of Cannabidiol on the Action Potentials of Ventricular Myocytes

In this set of experiments, patch-clamped ventricular myocyte were exposed to CBD while continuously monitoring their $V_{\text{rest}}$ and APs in current clamp mode. The generation of APs was stimulated by 0.9-1 nA depolarizing current pulses of 4 ms durations. Since the intracellular pipette solution did not contain Ca$^{2+}$-chelating agents, the generation of each AP was accompanied by a myocyte contraction. Therefore, current pulses were applied at a frequency of 0.2 Hz. During a typical experiment the following protocols were employed: first, whole-cell configuration was established and a 4 to 5 minute dialysis of the myocytes with a pipette solution was allowed to ensure an equilibrium of conditions between the pipette solution and the intracellular milieu. After achieving stable recordings of baseline electrical activity ($V_{\text{rest}}$ and AP parameters), myocytes were exposed to CBD for 5 to 10 minutes and was subsequently washed out.

The resting membrane potentials (mean ± SEM) were -77.4 ± 1.9 and -79.2 ± 1.7 mV in the control and after CBD treatment (n=9) myocytes, respectively (Figure 21A, inset and Figure 21B). Maximal amplitudes of AP and the rising rate of AP (maximal velocity; V/s, Figure 21C and Figure 21D) were not significantly altered in the presence of CBD. CBD (1 µM) consistently shortened the duration of AP (measured at 60 % of repolarization, APD$_{60}$) (Figure 21A inset and Figure 21E). Changes in AP shortening in response to CBD (1 µM) applications were noticeable within 20 to 30 seconds (Figure21A). Recoveries were usually partial and required a longer time.
Figure 21. Effect of cannabidiol on the excitability of ventricular myocytes. (A) Representative recordings show the APs in controls (dark grey area), in the presence of 1 μM CBD (light grey area) and after washout (striped area) in a ventricular myocyte; the insets on panel A show the time course of action potential duration (APD$_{60}$) and resting potential (V$_{rest}$) changes in response to CBD application (indicated by horizontal bars). (B-E) show summary of CBD effects on amplitude and shape of the AP in ventricular myocytes; quantification of the changes in V$_{rest}$(B), AP amplitude (C), AP maximal velocity(D) and AP duration (E), characterized by APD$_{60}$ in controls (dark grey bars) and in response to 1 μM CBD (light grey bars). Data are mean ± S.E.M., n = 6 – 9 cells from 6 to 9 for each group.
3.6 Effect of Cannabidiol on L-type Ca\(^{2+}\) Currents

We also investigated the effect of CBD (1 µM) on L-type Ca\(^{2+}\) currents. Figure 22A shows a typical record of L-type Ca\(^{2+}\) currents elicited by applying a single 300 ms voltage pulse to +10 mV from a holding potential of -50 mV in rat ventricular myocyte before and after a 10 minute superfusion with 1 µM CBD. The time frame of the effect of CBD on the density of L-type Ca\(^{2+}\) currents is shown in Figure 22B. The application of a vehicle (0.02% DMSO) for 10 minutes caused a 10-15% inhibition of the current density of L-type Ca\(^{2+}\) currents in experiments lasting up to 15 to 20 min. The effects of CBD were also investigated on the biophysical properties of L-type Ca\(^{2+}\) currents in rat ventricular myocytes. L-type Ca\(^{2+}\) currents were recorded in the presence of intracellular Cs\(^+\) and extracellular TEA\(^+\) to suppress K\(^+\) currents while retaining 95 mM Na\(^+\) in the extracellular solution. The elimination of contaminating Na\(^+\) current during the recording of L-type Ca\(^{2+}\) currents was achieved by applying voltage step-pulses from relatively depolarized \(V_h\) of -50 mV, which produced steady-state inactivation of sodium channels (Voitychuk, et al., 2012). As evident from the original recordings and \(I-V\) relationships (Figure 23A), L-type Ca\(^{2+}\) currents started to appear at \(V_m\) = -30 mV, reached a maximum at around \(V_m\) = +10 mV, and decreased at higher voltages approaching to zero at about \(V_m\) = +60 mV (Figure 23B). The maximal amplitudes of L-type Ca\(^{2+}\) currents were suppressed in the presence of CBD (1 µM).
Figure 22. Effect of cannabidiol on Ca$^{2+}$ currents mediated by L-type Ca$^{2+}$ channels in rat ventricular myocytes. (A) CBD inhibits L-type Ca$^{2+}$ currents recorded using whole-cell voltage-clamp mode of patch clamp technique. Current traces are recorded before (control) and after 10 min application of 1 μM CBD. L-type Ca$^{2+}$ currents were recorded during 300 ms voltage pulses to +10 mV from a holding potential of -50 mV. (B) Averages of the maximal currents of VGCCs presented as a function of time in the presence of vehicle (0.02% DMSO; filled circles) and 1 μM CBD (n=5 cells; open circles). Application time for the agents was presented in horizontal bars.

Figure 23. The effect of cannabidiol on voltage-current characteristics of L-type Ca$^{2+}$ channels in rat ventricular myocytes. (A) Representative recordings of L-type Ca$^{2+}$ currents in response to the depicted pulse protocol under control conditions and after application of 1 μM CBD. (B) Normalized and averaged I-V relationships of control L-type Ca$^{2+}$ currents (filled circles) and L-type Ca$^{2+}$ currents in the presence of 1 μM CBD (open circles) determined by applying a series of step depolarizing pulses from -70 mV to +70 mV in 10 mV increments for a duration of 300 ms. Data points (mean ± S.E.M.) are from 5 to 7 cells.
CBD did not change the steady-state activation curve of the L-type Ca\textsuperscript{2+} current (V\textsubscript{1/2} values in the control experiment and in the presence of CBD, they are -19.1 ± 0.4 mV and -17.6 ± 0.3 mV, respectively; n=6, Paired t-test, P>0.05) (Figure 24A). However, there was a hyperpolarizing shift of L-type Ca\textsuperscript{2+} currents in steady-state inactivation by 9.6 mV (i.e., from control value V\textsubscript{1/2} = -10.2 ± 0.9 mV to V\textsubscript{1/2} = -19.8 ± 1.1 mV in the presence of CBD) (Figure 24B). However, there was little effect on the slopes of the curves (k = 7.7 ± 0.5 mV and k = -4.5 ± 0.4 mV for the control activation and inactivation, respectively, versus k = 7.9 ± 0.3 mV and k = -4.2 ± 0.3 mV for the CBD-modified activation and inactivation, correspondingly).

In line with earlier reports (Soldatov, et al., 1998), kinetic analysis of L-type Ca\textsuperscript{2+} currents were titled to a double-exponential function with fast (τ\textsubscript{f}) and slow (τ\textsubscript{s}) inactivation time constants. Comparison of L-type Ca\textsuperscript{2+} currents in the absence and presence of CBD revealed noticeable acceleration of the current's inactivation kinetics by CBD. Quantification of the time constants of L-type Ca\textsuperscript{2+} currents inactivation showed that CBD (1 μM) significantly reduced τ\textsubscript{i} in the range of V\textsubscript{m} -30 mV and +10 mV (Figure 25).
Figure 24. Effect of cannabidiol on steady state activation and inactivation of L-type Ca2+ currents in rat ventricular myocytes. Steady-state activation (A) steady-state inactivation (SSI) (B) curves of L-type Ca2+ currents in the absence (filled circles) and presence of 1 μM CBD (open circles). Data are mean ± S.E.M, n= 5 cells are from 5 cells. Fit of experimental data points with Boltzmann equation.
Figure 25. Effect of cannabidiol on the kinetics of L-type Ca2+ currents in rat ventricular myocytes. Voltage-dependent fast (triangles) and slow (circles) inactivation time constants (τi) of L-type Ca2+ currents under control conditions (filled circles, and triangles) and in the presence of 1 μM CBD (open circles and triangles). Data are mean ± S.E.M., n= 5-6 cells.
Chapter 4: Discussion

Our results show that CBD has a negative inotropic effect on the contractility of rat ventricular myocytes. The negative inotropic effect of CBD appears to be mainly due to impaired Ca\(^{2+}\) signaling in cardiomyocytes. Furthermore, the results indicate that the impaired Ca\(^{2+}\) signaling emanates from the inhibitory effects of CBD on the function of L type Ca\(^{2+}\) channel.

Actions of CBD on the cardiovascular system influences multiple organ systems and involves complex cellular and molecular mechanisms (see Baranowska-Kuczko, MacLean, Kozlowska and Malinowska, 2012; Stanley et al., 2013). CBD has been shown to have various effects on muscular structures, neuronal and endothelial cells and alter the activities of several receptors and ion channels directly (see Hejazi et al., 2006; Pertwee 2008; 2010). Furthermore, other factors such as the activation of autonomic reflexes, the presence of endothelial cells and fatty-acid based metabolic products have been reported to contribute to the complexity of CBD actions on the heart (Pertwee 2008; Stanley et al., 2013). On the other hand, acutely dissociated ventricular myocytes have several advantages over in-vivo and traditional in-vitro conditions, since it excludes some of the factors mentioned above and also the influences of reflex pathways, autonomic nerve endings, neurotransmitter uptake systems, gap-junction connections and coronary perfusion status.

In our experiments, using video edge detection techniques in individual myocytes, it was found that CBD causes a significant reduction in the maximal amplitude of shortening (expressed as a percentage of resting cell length) without altering the time frame (such as TPK shortening and THALF relaxation) of myocytes shortening. Our
findings from myocyte shortening and intracellular Ca\textsuperscript{2+} measurement experiments are in agreement with earlier studies reporting that CBD exerts a negative-inotropic effect in isolated-cardiac muscle preparations (Smiley, Karler and Turkanis, 1976; Nahas and Trouve, 1985). Furthermore, results from our study suggest that a direct interaction of CBD with ventricular myocytes, rather than the actions of CBD on nerve endings and neurotransmitter uptake systems are mainly responsible for the negative inotropic effects of CBD in the heart.

The negative-inotropic actions of CBD might be attributed to the impaired release of Ca\textsuperscript{2+} from the SR. In fact, CBD and other cannabinoids have been reported to modulate the ryanodine sensitive intracellular Ca\textsuperscript{2+} stores in neurons (Drsydale et al., 2008) glia (Mato et al., 2010; Pertwee et al., 2010). However, the amplitude and kinetics of caffeine-induced Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores were not changed by CBD suggesting that ryanodine-sensitive intracellular Ca\textsuperscript{2+} stores are not involved in the negative-inotropic effects of CBD observed in this study.

Cannabidiol can alter the levels of second messengers such as cAMP, cGMP and protein kinase C which are known to be involved in tuning the Ca\textsuperscript{2+} sensitivity of the contractile proteins. In an earlier study of cardiac muscle membranes, cAMP levels have been shown to be unaltered in the presence of CBD (Hillard, Pounds, Boyer and Bloom, 1990). Furthermore, the sensitivity of contractile proteins to intracellular Ca\textsuperscript{2+} remained unchanged in the presence of CBD suggesting that phosphorylation and de-phosphorylation of the contractile proteins did not play a significant role in the negative-inotropic action of CBD. Collectively, these results suggest that the effects of CBD on myocyte contractility are not related to changes in intracellular Ca\textsuperscript{2+} release machinery or
sensitivity of myofilaments to Ca\(^{2+}\). In addition, in the presence of CBD, resting levels of intracellular Ca\(^{2+}\), and cell length of ventricular myocytes, remained unaltered suggesting that CBD does not significantly affect Ca\(^{2+}\) homeostasis under resting conditions.

During excitation-contraction coupling, alterations in the amplitudes and kinetics of the cardiac action potential (AP) are closely associated with corresponding changes in the contractility of myocytes. In our study, cardiac APs were recorded using the whole cell patch clamp technique. It was found that the amplitudes and dV/dt\(_{\text{max}}\) of APs were not altered in the presence of CBD suggesting that voltage-gated sodium channels are not affected by CBD. For this reason, the effect of CBD on the functional properties of voltage-gated sodium channels was not investigated any further. However, the decrease of AP duration, and suppression of the second phase of the AP was evident in whole cell recordings from cardiomyocytes. Since the plateau (phase 2) of AP is mainly due the influx of Ca\(^{2+}\) through L-type voltage-gated Ca\(^{2+}\)channels, these results suggest that CBD can act on L-type Ca\(^{2+}\) channels. Indeed, whole cell recordings of Ca\(^{2+}\) currents in cardiomyocytes, in agreement with the recordings of APs, indicated that CBD inhibits potently the function of L-type voltage-gated Ca\(^{2+}\) channels with an IC\(_{50}\) value of 0.6 µM. An analysis of current kinetics indicated that the inactivation of Ca\(^{2+}\) currents can double the exponential decay and CBD can decrease the amplitudes of the Ca\(^{2+}\) currents by accelerating both fast and slow components of the inactivation process. The effects of CBD on the gating characteristics of Ca\(^{2+}\) channels were further investigated by studying the activation and inactivation curves in the absence and presence CBD. Although half activation voltage and the slope of the activation curve were not altered, there was a
hyperpolarizing-shift of the inactivation curve further suggesting that CBD changes the voltage-dependent inactivation process.

Collectively, these results suggest that during excitation-contraction coupling, shortening of action potential due to the inhibition of L-type Ca\(^{2+}\) channels decreases Ca\(^{2+}\)-induced Ca\(^{2+}\) release from SR and causes the negative-inotropic effect of CBD observed in our experiments and reported in earlier studies. In line with these findings, although caffeine induced contractures and myofilament sensitivity to Ca\(^{2+}\) remained unchanged, electrically-induced Ca\(^{2+}\) transients were significantly depressed by CBD; further suggesting that Ca\(^{2+}\)-induced Ca\(^{2+}\) release was impaired in the presence of CBD. In earlier studies CBD has been reported to inhibit the uptake of dopamine and other neurotransmitters (Poddar and Dewey, 1980; Pandolfo, et al., 2011). However, an uptake mechanism is not likely to be involved in our studies on isolated ventricular myocytes. Furthermore, inhibition of uptake mechanisms for dopamine and/or catecholamines such as epinephrine and norepinephrine would lead to well-established positive inotropic actions, rather than a negative inotropic effect as observed in the present study.

Although CBD does not activate known cannabinoid receptors, some of its pharmacological actions have been shown to be reversed by cannabinoid receptor antagonists (Pertwee, 2008). Recent studies have shown that the antagonist sensitivity of CBD effects is due to the inhibition of fatty acid amide hydrolase activity, an enzyme that inactivates endogenous cannabinoids and related to subsequent increases in tissue endocannabinoids levels (Pertwee, 2010). However, both amplitudes of myocyte shortening and the L-type Ca\(^{2+}\) currents were not altered by URB597, a potent inhibitor
of fatty acid amide hydrolase (Al Kury et al., 2014), suggesting that the activity of increased fatty acid amide hydrolase was not involved in the actions observed in CBD.

The interaction between L-type Ca\textsuperscript{2+} channels and CBD has not been studied in detail. In an earlier study, \textsuperscript{45}Ca\textsuperscript{2+}-uptake in brain synaptosomes had been shown to be inhibited by CBD (Harris and Stokes, 1982). Similarly, CBD induced increases in intracellular Ca\textsuperscript{2+} levels were found to be sensitive to blockers of L-type Ca\textsuperscript{2+} channels (Drysdale et al., 2006). In agreement with these results, contractions of smooth muscles elicited by high-K\textsuperscript{+}-containing solutions were also significantly inhibited by CBD (Cluny, Naylor, Whittle and Javid, 2011). The interaction between T-type Ca\textsuperscript{2+} channels and CBD was investigated in a recent study, on human CaV3 channels stably expressed in human embryonic kidney 293 cells and T-type channels in mouse sensory neurons (Ross et al., 2008). At moderately hyperpolarized potentials, CBD inhibited peak CaV3.1 and CaV3.2 currents with IC\textsubscript{50} values of 1 \(\mu\text{M}\) but was less potent on CaV3.3 channels. CBD inhibited sensory neuron T-type channels by about 45\% at 1 \(\mu\text{M}\). However, in recordings made from a holding potential of -70 mV, 100 nM CBD inhibited more than 50\% of the peak CaV3.1 current. CBD produced a significant hyperpolarizing shift in the steady state inactivation potentials for each of the CaV3 channels (Ross et al., 2008). In this respect our results indicate for the first time that CBD inhibits the function of L-type Ca\textsuperscript{2+} channels in ventricular myocytes.

In clinical studies, it has been shown that acute CBD intake does not cause a significant change in blood pressure and heart rate (Stanley, 2013a). However, several earlier studies indicate that increases in blood pressure and heart rate during stressful conditions are markedly attenuated by CBD (Gomes, et al., 2013; Granjeiro, Gomes,
Guimaraes, Correa and Resstel, 2011; Resstel, Joca, Moreira, Correa and Guimaraes, 2006; Resstel, et al., 2009). In fact, in in vivo studies, it has been demonstrated that increased heart rate and blood pressure, one of the most consistent effects of cannabis intoxication, are significantly decreased by CBD (Nahas and Trouve, 1985). These studies suggest that CBD can exert negative inotropic and chronotropic actions under certain clinical conditions such as stress and cannabis intoxication.

CBD, in the concentration range used in this study (0.1-10 µM), has been shown to modulate functions of various receptors and ion channels such as Ca$^{2+}$ channels (Ross et al., 2008), 5-HT3 (Yang et al., 2010), and nicotinic receptors (Mahgoub et al., 2013). The peak plasma concentrations of CBD in healthy volunteers following administration of Sativex™ (1:1 ratio of Δ9-THC and CBD) was reported to be between 0.01µM to 0.05µM (Massi, et al., 2013). In earlier clinical studies, mean CBD levels of 0.036 μM were determined in blood analysis following a 6-week oral treatment with CBD at doses of 10 mg/kg/day (Consroe et al., 1991). However, CBD is a highly lipophilic compound and easily passes biological membranes and accumulates in tissues and it can reach to several fold higher tissue concentrations than in blood. Collectively, these findings suggest that CBD, at relatively high concentrations, can suppress the function of the cardiovascular system. In fact, cardiac failure due to depressed heart contractility has been suggested to be the main cause of mortality through cannabis intoxication (Bergamaschi et al., 2011). Thus it is likely that some of the effects of the cannabis plant are mediated by CBD during cannabis intoxication.
In addition to cardiac contractility, the electrical activity of heart has also been suggested to be affected by CBD. In an earlier study, CBD has been shown to have beneficial actions in ischemia-induced cardiac arrhythmias (Walsh et al., 2010). The shortening of the AP duration by CBD observed in our study can be beneficial or harmful, depending on the underlying pathology. Thus, during acute ischemia, in which the duration of the cardiac APD is already shortened, a further decrease should be proarrhythmic (Den Ruijter et al., 2007). However, shortening of AP duration should be beneficial in preventing those arrhythmias caused by triggered activities observed in conditions such as heart failure (Den Ruijter et al., 2007). In conclusion, the results indicate for the first time, that CBD inhibits myocyte contractility by acting on L-type Ca\(^{2+}\) channels.
Chapter 5: Limitation and Future Directions

The scope of this research is mainly concerned with the effects of CBD on healthy ventricular cells, although endocannabinoids are stress induced neurotransmitters. In addition to the effects of CBD on L-type calcium channels its effect on NCX should also be established due to their influence on cytosolic calcium levels and the viability of ventricular cells in the presence of CBD.

Future research may also include expressing calcium channels onto HEK-293 cells, this allows us to exclude or include the indirect effects of CBD on intracellular signaling that can affect calcium channels. Another method is to express the calcium channel each subunit at a time and find where CBD exerts its effects most effectively and thereby establish the mechanism by which CBD exerts its effects.
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