STUDIES INVESTIGATING THE MECHANISMS OF THE CARDIOPROTECTIVE EFFECTS OF CANNABIDIOL

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Declaration

The thesis in candidature for the degree of Doctor of Philosophy has been composed entirely by myself. The work which is documented was carried out by myself. All sources of information contained within which have not arisen from the results generated have been specifically acknowledged.

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Publications

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Abstract for oral communication

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Abstract

Claire Y Hepburn.
Studies investigating the mechanisms of the cardioprotective effects of CBD.
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The phytocannabinoid cannabidiol (CBD) has a complex pharmacology which is thought to include, but is not limited to, an ability to act as an inverse agonist at the CB_1 and CB_2 receptors and an antagonist of GPR55. Moreover, is has been shown to reduce infarct size and ameliorate reductions in left ventricular function *in vivo*. These improvements in the pathogenesis of experimental MI are accompanied by a reduction in inflammatory cell migration to the area at risk. More recently it has been shown that CBD is anti-arrhythmic in acute experimental MI. Thus, it was suggested that the cardioprotective effects of CBD might be due to an anti-inflammatory action. In addition, GPR55 receptor activation is acknowledged to mediate mobilisation of intracellular Ca^{2+} (Ca^{2+}_i) which could potentially be pro-arrhythmic and so CBD, as an antagonist may confer cardioprotection via GPR55. However, the receptors and/or mechanisms responsible for mediating the cardioprotective effects of CBD are get to be determined. The present studies were therefore performed to; (1) better understand the pharmacology of CBD by assessing haemodynamic responses to CBD and other cannabinoids ligands in anaesthetised rats, (2) investigate the receptors involved in the anti-arrhythmic effect of CBD in a rat model of coronary artery occlusion (CAO), and (3) investigate if CBD can alter $[Ca^{2+}]_i$ in isolated rat cardiomyocytes. The characterisation of the pharmacology of CBD *in vivo* showed that; firstly, CB_1 receptor activation causes a hypotensive response which can be dose-dependently inhibited by AM251; secondly, both CBD and AM251 alone (a CB_1 receptor antagonist and GPR55 agonist) can induce vasodepressor responses and finally, CBD can potentiate the AM251-mediated hypotension when co-administered, suggesting possible cross-talk between the CB_1 and GPR55. Results from CAO studies showed that CBD and AM251 each have the capacity to reduce arrhythmias. Moreover, when CBD and AM251 were co-administered the anti-arrhythmic capacity of either alone was potentiated. However, the degree of potentiation was dependent on the order of administration, suggesting that more than one receptor is involved in the summative anti-arrhythmic effects. The investigation of cardiomyocyte $[Ca^{2+}]_i$ suggested that AM251 can modulate $[Ca^{2+}]_i$ at the level of the cardiomyocyte, while CBD cannot. These data give novel insight into the anti-arrhythmic effects of CBD and, moreover, for the first time demonstrate that AM251 is anti-arrhythmic. In addition, these data suggest a role for GPR55 in increasing $[Ca^{2+}]_i$ via AM251.

Keywords: Myocardial Ischaemia, Arrhythmias, Cannabinoids, Cannabidiol, GPR55, CB_1 receptor.

Abbreviations

AAC	Area above the curve
Abn-CBD	Abnormal cannabidiol
AEA	Anandamide
ACEA	Arachidonyl-2'-chloroethylamide
AF	Atrial fibrillation
Akt	Protein kinase B
AM	Acetoxymethyl
AM251	*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide
AM630	6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
AUC	Area under the curve
AV	Atrioventricular
BP	Blood pressure
BPM	Beats per minute
C1-6	Complement proteins 1-6
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Cytosolic free/intracellular calcium concentration
$CB_{1/2}$	Cannabinoid receptor 1/2
CBD	Cannabidiol
CHO	Chinese hamster ovary
CICR	Calcium-induced calcium release
CO_2	Carbon dioxide
CP 55,940	(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol
CNS	Central nervous system
CsA	Cyclosporin A
CVD	Cardiovascular disease
CyP-D	Cyclophilin-D
DAD	Delayed afterdepolarisation
DHP	1,4-dihydropyridine

DMSO	Dimethyl sulfoxide
DVA	Delayed ventricular/phase Ib arrhythmia
ECG	Electrocardiogram
ECS	Endocannabinoid system
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
ETC	Electron transport chain
EtOH	Ethanol
FAAH	Fatty acid amide hydrolase
GPCR	G-protein coupled 7-transmembrane spanning receptors
GPR55	G protein-coupled receptor 55
H^+	Hydrogen ion
HCN	Hyperpolarisation-activated cyclic nucleotide-gated channels
HEK293	Human embryonic kidney 293
HR	Heart rate
HRP	Horseradish peroxidise
H_2O_2	Hydrogen peroxide
$I_{Ca,L}$	L-type Ca^{2+} channel current
$I_{Ca,T}$	T-type Ca^{2+} channel current
IHC	Immunohistochemistry
I_{K1}	Delayed rectifier potassium current
IL-1β	Pro-interleukin-IL-1β
IMM	Inner mitochondrial membrane
IPC	Ischaemic preconditioning
IP_3	Inositol 1,4,5-triphosphate
IP_3R	Inositol 1,4,5-triphosphate receptor
I/R	Ischaemia and reperfusion
ir.VF	Irreversible ventricular fibrillation
I_{TI}	Transient inward current
I.V.	Intravenous
IVA	Immediate ventricular/phase Ia arrhythmia
K^+	Potassium ion
$[K^+]_e$	Extracellular K^+ ion concentration
KCl	Potassium chloride
LAD	Left anterior descending
Late I_{Na}	Inward sodium current
LPC	α-lysophosphatidylcholine

LPI	Lysophosphatidylinositol
LPMI	Loss of plasma membrane integrity
LTCC	L-type calcium channel
MABP	Mean arterial blood pressure
MAC	Membrane attack complex
MAGL	Monoacylglycerol lipase
MAPK	Mitogen activated protein kinase
mESPCs	Miniature excitatory postsynaptic currents
Mg^{2+}	Magnesium ion
MI	Myocardial ischaemia
MPTP	Mitochondrial permeability transition pore
n	Number of replicates
NA	Noradrenaline
Na^{+}	Sodium ion
$[Na^{+}]_i$	Intracellular sodium ion concentration
NaCl	Saline
NAD^{+}	Nicotinamide adenine dinucleotide
NADA	N-arachidonoyl dopamine
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFT	Nuclear factor of activated T cells
NCX	Na^{+}-Ca^{2+} exchanger
NF $_{\kappa}$B	Nuclear factor kappaB
NMDA	N-methyl-D-aspartate
O^{-}_{2}	Superoxide anion
OMM	Outer mitochondrial membrane
O-1602	5-Methyl-4-[(1*R*,6*R*)-3-methyl-6-(1-cyclohexen-1-yl]-1,3-benzenediol
PBS	Phosphate buffered saline
PAF	Platelet activating factor
PARP	Poly(ADP-ribose) polymerase
PINK1	PTEN (phosphatase and tensin homologue on chromosome 1)
PI3K	Phosphatidylinositol-3-OH kinase
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor gamma
RGB	Red-green-blue
RISK	Reperfusion injury salvage kinase
ROS	Reactive oxygen species
r.VF	Reversible ventricular fibrillation

RyR	Ryanodine receptor
SA	Sinoatrial
SEM	Standard error of the mean
SERCA	Sarcoplasmic reticulum calcium transport ATPase
SfA	Sanglifehrin A
SR	Sarcoplasmic reticulum
SR141617A	*N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (Rimonabant)
Syk	Spleen tyrosine kinase
TNFα	Tumour necrosis factor α
TPR	Total peripheral resistance
TRPV1	Transient receptor potential vanilloid 1 channel
VCAM	Vascular cell adhesion molecule
VDAC	Voltage-dependent anion channel
VF	Ventricular tachycardia
VPB	Ventricular premature beat
VT	Ventricular fibrillation
WIN-55,212-2	(*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate
WT	Wild-type
$\Delta\psi_m$	Mitochondrial membrane potential
Δ^9-THC	(-)-Δ^9-tetrahydrocannabinol
2-AG	2-arachidonyl glycerol
$5HT_3$	5-hydroxytryptamine (serotonin) receptor 3

1. General Introduction

1.1 Prevalence of cardiovascular disease

Diseases of the heart and circulatory system are known generically as cardiovascular disease (CVD) and this terms encompasses myocardial infarction, angina pectoris, heart failure and many more conditions (Roger *et al*., 2012). Each year CVD is the principle cause of mortality in Europe and accounts for the deaths of approximately 4.1 million individuals each year in Europe alone (as referenced in Nichols *et al*., 2013). Moreover, CVD is not a disease which manifests only in old age, it accounts for 31% deaths before 65 in men and 27% of deaths before 65 in women in Europe (as referenced in Nichols *et al*., 2013). The financial burden of CVD in Europe runs to €196 billion a year (as referenced in Nichols *et al*., 2013). Therefore, the prevention and improved management of CVD remains a prominent concern of governments and health care professionals not only across Europe but across the globe.

CVD also includes disorders of cardiac rate or rhythm, arrhythmias, (Levick, 2003) and incidences of atrial fibrillation (AF) are increasing (Go *et al*., 2001; Deo and Varosy, 2012), in addition, no single treatment has been found that can alone mitigate the effects of AF (Deo and Varosy, 2012) or ventricular arrhythmias (Della Bella *et al*., 2013). As such, any emerging strategies which can help to abate the burden arrhythmias and CVD would likely be of significant interest.

1.2 Physiology of the heart

The primary function of the cardiovascular system is to maintain homeostasis within the entire body; this is achieved by efficient provision of oxygen and nutrients and removal of products of cellular metabolism from tissues. The heart, a double pump that moves blood around the systemic and pulmonary circulations, meets these criteria.

The impulse-conducting system of the heart is responsible for initiating and maintaining synchronous pumping of the heart. The impulse-conducting system consists of the sino-atrial (SA) node, atrioventricular (AV) node and the His-Purkinje system; the electrophysiological properties of each of the components of the impulse-conducting system differs which allows for staggered activation of myocardial contraction as this permits most efficient pumping.

1.2.1 Electrophysiological properties of the myocardium

Synchronous contraction of the heart relies upon ordered transmission of electrical impulses through the impulse-conducting system. There are three types of cells in the cardiac tissue capable of electrical excitation: the pacemaker cells which are those that are found in the SA and AV nodes, specialised tissues of the impulse-conducting system (the His-Purkinje system) and, finally, the ventricular and atrial muscle cells.

1.2.1.1 SA node

Under physiological conditions there is a slow depolarisation during phase 4 of the action potential in the SA node. This results in spontaneous depolarisation of SA nodal cells, without external activation, which subsequently drives depolarisation of the cells downstream in the impulse-conducting system and ultimately dictates heart rate (Satoh, 2003; Mangoni and Nargeot, 2008). The genesis of automatic electrical excitation is dependent upon diastolic depolarisation following repolarisation, a process that drives the next action potential (Mangoni and Nargeot, 2008). The resting membrane potential of the SA node is inherently unstable; three principle time-dependent, ionic channel currents bring about its decay (Figure 1.1; Satoh, 2003):

1. A small inward flow of sodium (Na^+) ions is provided by the funny (I_f) current by way of the HCN2 and HCN4 subunits of the hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels (Bucchi *et al*., 2012). Expression of these subunits is significantly higher in the SA node than in the wall of the right atrium free wall (Yeh *et al*., 2009). Pharmacological blockade of this current slows heart rate and is used clinically in the treatment of angina and heart failure, this is only activated by hyperpolarisation (DiFrancesco and Camm, 2004; Bucchi *et al*., 2012).
2. The I_k current is a slowly decaying, outward potassium (K^+) current. Rapidly (I_{Kr}) and/or slowly (I_{Ks}) activating channel currents contribute the delayed K^+ efflux in nodal cells. The contribution of these two currents to spontaneous depolarisation in the SA nodes shows variability between species. For example, in the rat SA node, the I_{Kr} blocker E-4031 can suppress SA nodal cell spontaneity however, a similar effect cannot be achieved with a I_{Ks} blocker (Shinagawa *et al*., 2000; Lei *et al*., 2001). In contrast, in guinea pig nodal cells both I_{Kr} and I_{Ks} currents play an equal role in contributing to spontaneous depolarisation (Matsuura *et al*., 2002).

3. The T-type Ca^{2+} channel ($I_{Ca,T}$) and the L-type Ca^{2+} channel ($I_{Ca,L}$) currents contribute to depolarisation, the latter current when the membrane potential reaches values of ~-55mV; this current is the one that provokes action potential formation (Levick, 2003; Mangoni and Nargeot, 2008). The α subunits predominantly expressed in the SA node are $Ca_v3.1$ and $Ca_v3.2$ (Cribbs *et al.*, 1998, Satoh, 2003) for the $I_{Ca,T}$ current and $Ca_v1.2$ for the high-voltage activated, $I_{Ca,L}$ channel (Bohn *et al.*, 2000, Satoh, 2003). The contribution of the $I_{Ca,T}$ to the SA spontaneous AP is thought to be small on the basis that the activation threshold for this current is similar to the I_{Na} channel (Satoh, 2003).

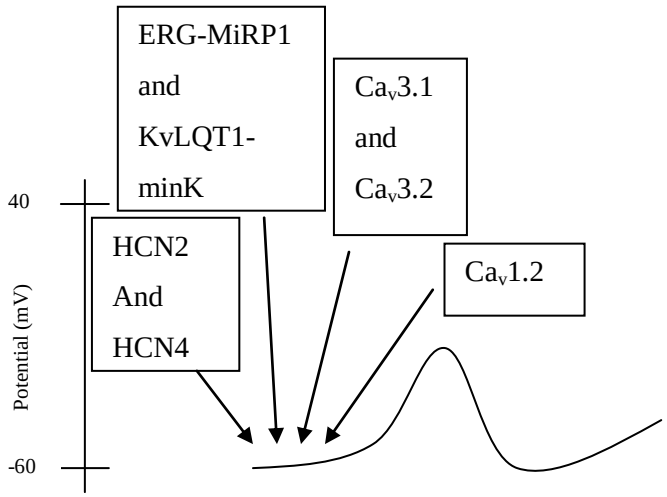

Figure 1.1 Typical SA node action potential which illustrates the channels responsible for spontaneous depolarisation. Adapted from Levick, (2003).

There is a degree of variability between species in the currents involved in the development of action potentials. For example, in the guinea pig a rapidly activating K^+ current (I_{kr}) and a sustained inward current (I_{st}) have been shown to also contribute to the spontaneity of the SA nodal cells. In the monkey the inwardly rectifying K^+ (I_{k1}) with a reduced I_f current contributes to SA node spontaneity (Guo et al., 1997; Shinagawa et al., 2000; Mitsuiye et al., 2000; Satoh, 2003).

The automaticity exhibited by the SA node propagates action potential generation elsewhere in the impulse conducting system, including the ventricular myocytes. The action potential of the ventricular

myocytes differs in waveform morphology from those of the automatic cells of the heart. The five phases of ventricular cell depolarisation are illustrated in Figure 1.2:

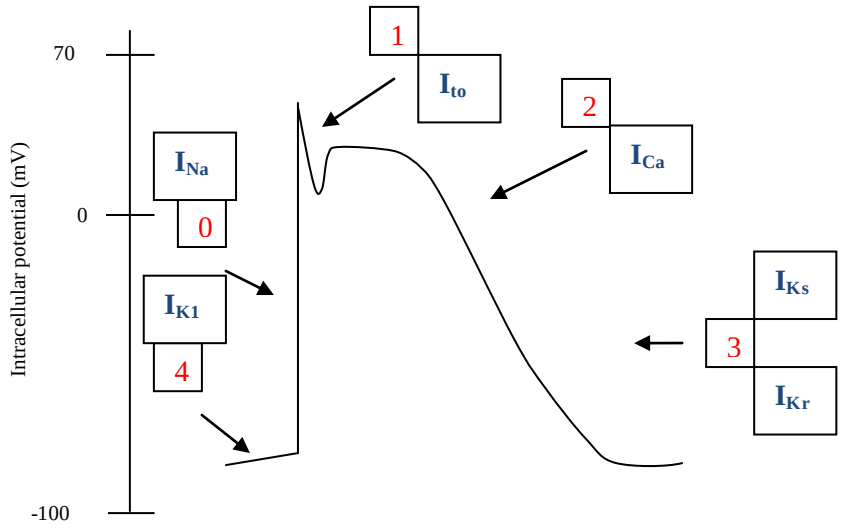

Figure 1.2 An illustration of a ventricular myocyte action potential. The channels which generate each phase are detailed in blue, phase numbers in red. Adapted from Nattel and Carlsson, (2006) as referenced by Grant, (2009).

- Phase 0: This phase is characterised by rapid depolarisation with reversal of the membrane potential. The I_{Na} current is responsible for this phase, with activation occurring at around -60mV.

- Phase 1: Phase 1 brings about early repolarisation of the overshoot caused during phase 0. The channels, which bring about this phase carry the transient outward K^+ (I_{to}) current and open transiently in response to rapid depolarisation of the cell membrane. This phase determines the activation of the Ca^{2+}-dependent ion channels and thereby its physiological function appears to be to modulate the plateau phase of the action potential. Moreover, in rats who exhibit high $I_{to,}$ there is a propensity for action potentials to be extremely short and there is lack of a distinct plateau (Gussak *et al.*, 2000; Niwa and Nerbonne, 2010). There are both rapid ($I_{to,f}$) and slow ($I_{to,s}$) facets of this current and the expression of each varies across the myocardium (Oudit *et al.*, 2001). K_v4.2/4.3 subunits are responsible for $I_{to,f}$ and K_v1.4 subunits drive the function of $I_{to,s}$ (Niwa and Nerbonne, 2010).

- Phase 2: Unique to the cardiac action potential, this phase is typified by the inward current I_{Ca}. This phase is crucial in prevention of rapid repolarisation (Bers and Guo, 2005). Ca^{2+} entry in this phase is facilitated by L-type Ca^{2+} channels (LTCC; $Ca_v1.2$) and to a lesser extent, T-type Ca^{2+} channels ($Ca_v3.1$ and $Ca_v3.2$) and the influx of Ca^{2+} facilitates calcium release from the sarcoplasmic reticulum (SR); this process consequently causes contracture of the myocyte (Grant *et al*., 2009; Meza *et al.*, 2013).

- Phase 3: After the plateau of phase 2 has completed, the repolarisation phase begins to dominate ion movement once again. Repolarisation is the process of time-dependent increase in the conductance of K^+ and is induced by opening of the delayed inward rectifier K^+ channels, of which two distinct channels, I_{Kr} and I_{Ks}, carry rapid and slow rectification currents, respectively (Natell, 2008).

- Phase 4: This phase is termed the resting phase and it is the function of the inward rectifier channel K^+ current (I_{K1}) during this phase that sets the resting membrane potential of the subsequent action potential. The I_{K1} current in ventricular myocytes is a composite of Kir2.1 and Kir 2.2 voltage-dependent ion channels, and ultimately determines terminal repolarisation (Biliczki *et al*., 2002; Nagy *et al*., 2013).

The intensity of the rapid upstroke and plateau are readily influenced by the transmembrane potential at depolarisation (Gettes and Reuter, 1974). The contraction of cardiac myocytes transpires as a result of transient changes in cytosolic calcium concentration; this is directly modified upon transmembrane depolarisation (Stern and Lakatta, 1992).

1.2.2 Excitation-contraction coupling

Excitation-contraction coupling is the process that occurs in myocytes whereby action potentials are translated into activation of the contractile machinery. This process is reliant on transient changes in intracellular calcium (Ca^{2+}) ion concentrations and it is critical for guaranteeing the synchronous and timely contraction of the heart as a whole (Stern and Lakatta, 1992).

In physiological conditions action potentials in cardiomyocytes are initiated by the pacemaker cells of the SA node, this stimulates the cell interior to depolarise concurrently opening voltage-sensitive, membrane-bound, LTCC. Ca^{2+} ions enter the cell interior down their concentration gradient and induce intracellular Ca^{2+} ion mobilisation from the SR; this process termed calcium-induced calcium release

(CICR). The free cytosolic Ca^{2+} then binds to the troponin C molecules on the actin-troponin-tropomyosin complex. This induces a conformational change in the troponin C that results in revelation of the myosin binding site on the actin filaments. A complex series of protein interactions then occurs, terminating in formation of an actin-myosin cross-bridge which facilitates the contractile cycle of the myocytes (Levick, 2003; Stern and Lakatta, 1999).

1.3 Myocardial ischaemia

Myocardial ischaemia (MI) is the process whereby occlusion of a coronary artery causes insufficient provision of oxygen and nutrients and deficient removal of metabolic waste products from cardiac myocytes. The early consequences of ischaemia are metabolic changes accompanied by deterioration in ion homeostasis and consequent electrophysiological changes. Late changes include alterations in contractile function and induction of cellular necrosis (Carmeliet, 1999).

1.4 Manifestations of ischaemia

The manifestations of ischaemia can be immediate or delayed. The immediate changes occur within minutes of occlusion and include depletion of energy-rich phosphate stores. The depleted adenosine triphosphate (ATP) concurrently leads to ion derangements, all of which contributes to loss of normal contractile function and generation of arrhythmias.

1.4.1 Depleted energy generation

A prolonged reduction in coronary blood flow perturbs substrate metabolism in the myocardium and MI is associated with both a reduction in fatty acid oxidation and an increase in glycolysis (Knuuti and Tuunanen, 2010). This in turn reduces the production of energy-rich phosphates like ATP (Jaswal *et al*., 2011), which causes reduced tension generation in the myocardium with a concurrent decrease in stroke volume. A cascade then occurs involving loss of ion homeostasis and electrical inhomogeneity and cardiovascular performance is consequently compromised. In addition, the acute reduction in ATP causes an alteration in ion homeostasis and leakage of K^+ and Ca^{2+} across the myocardial cell membrane, thus perturbing the electrophysiological properties of the cardiomyocyte. The rising concentrations of purine precursors, which arise as a result of failed ATP generation, are degraded to

xanthine and hypoxanthine. These metabolites are substrates for xanthine oxidase, a superoxide producer, during ischaemia (Maxwell and Lip, 1997).

1.4.2 Perturbation of normal ion balance

Augmented oxidative phosphorylation causes ion concentrations to be disturbed with Na^+, K^+, Ca^{2+} and hydrogen (H^+) being the principle victims. Derangement of ion homeostasis can cause immediate cell death if extreme and can further impede recovery of those cells that have incurred non-fatal injury (Carmeliet, 1999; Piper, Meuter and Schafer, 2003).

1.4.2.1 Na^+

As a result of decreased energy generation during ischaemia, intracellular Na^+ concentrations [Na^+] are amplified. These changes are brought about by impaired function of the Na^+ channels which cause a persistent inward Na^+ current (late I_{Na}) coupled with reduced active outward flow (Hoyer *et al*., 2011). Although the late I_{Na} is present in healthy myocytes and contributes to normal Na^+ accumulation, it is the incomplete block of Na^+/K^+-ATPase, caused by the reduction in energy generation upon occlusion, which induces reduced removal of Na^+ ions from the cytosol. Moreover oxidative stress from oxygen free radicals has been implicated as a factor responsible for pump inhibition (Soliman *et al*., 2012). This accumulation is worsened still by acidosis. Acidosis induces the Na^+/H^+ exchanger to move H^+ across the cell membrane to the cell exterior concomitantly moving Na^+ into ischaemic myocytes (Carmeliet, 1999). Previous studies have shown that blockade of this exchanger can reduce Na^+ accumulation post-occlusion, substantiating this hypothesis (Anderson *et al*., 1991; Pike *et al*., 1993; Carmeliet, 1999). In a bid to counter the accumulation of intracellular Na^+ and hence depolarisation, the Na^+/Ca^{2+} exchanger is reversed (Soliman *et al*., 2012). The prevention of Na^+ overload inadvertently triggers Ca^{2+} overload, a development that induces contracture and thus myocellular damage but it is also proarrhythmic (Carmeliet, 1999; Piper, Meuter and Schafer, 2003). Reduction of the late I_{Na} in isolated ventricular myocytes can suppress arrhythmic activity, for example, delayed after depolarisations (DADs) and triggered activity (Song, Shryock and Belardinelli, 2008). Furthermore, blocking this current in the isolated guinea pig heart caused a reduction in energy loss and an improvement of contractile function (Hoyer *et al*., 2011).

1.4.2.2 K^+

Within minutes of cessation of coronary blood flow ischaemic cells within the ischaemic zone depolarise (Janse and Kleber, 1981). This depolarisation is directly attributable to extracellular accumulation of K^+ ions whose concentrations can exceed 20mmol l^{-1} (Janse and Kleber, 1981; Janse and Wit, 1989; Wilde and Aksnes, 1995; Carmeliet, 1999; Miura *et al*., 2012). The build up of extracellular K^+ $[K^+]_e$ during ischaemia typically occurs in three phases; a rapid accumulation occurring within the first 20 seconds, a plateau which occurs during 3 to 10 minutes post-occlusion, and a second accumulation which occurs more slowly during the period 15 to 30 minutes post-occlusion (Carmeliet, 1999).

Under physiological conditions there is a passive K^+ efflux from myocytes, which is actively balanced by K^+ uptake by the Na^+/K^+ pump; ischaemia disturbs this homeostatic mechanism (Carmeliet, 1999). There are three prime factors proposed to be responsible for perturbation of K^+ homeostasis, these are; altered K^+ efflux, diminished K^+ influx and osmosis. Accumulation of metabolic waste products alters the osmotic balance across the cell membrane of cells in the ischaemic zone, which concurrently causes water to move from the extracellular to intracellular spaces, decreasing the volume of the extracellular space. Accumulation of osmotically charged particles in the cytosol of ischaemic myocytes encourages K^+ loss to the extracellular environment. The Na^+/K^+ pump is responsible for the maintenance of K^+ homeostasis in physiological conditions. Upon coronary occlusion the function of this ATP-requiring pump diminishes as intracellular ATP levels are depleted. A direct consequence of deteriorating Na^+/K^+ pump function is an increase in $[K^+]_e$ due to the passive K^+ efflux from the cell not being actively matched. The weakening of pump activity is by no means extreme but is adequate to perturb normal K^+ ion balance. Interestingly, free radicals have been implicated in depression of pump activity in addition to the ATP-related effects (Matsuura and Shattock, 1991; Carmeliet, 1999). The final mediator of $[K^+]_e$ accumulation is proposed to be increased K^+ efflux. Ultimately this increased K^+ efflux will cause cardiomyocytes to depolarise (Carmeliet, 1999).

The elevated $[K^+]_e$ can acutely affect the electrophysiological properties of the ischaemic myocytes. As mentioned above, a direct result of ischaemia is depolarisation of those cells in the ischaemic zone with concomitant reduction in amplitude of cardiac action potentials. In addition, action potentials of reduced size and duration are observed. Together these factors favour the occurrence of reentry-induced arrhythmias (Carmeliet, 1999). Moreover, the electrophysiological perturbations of ischaemia can be mimicked simply by an increase in $[K^+]_e$ (Hill and Gettes, 1980; Hirche *et al*., 1980). More recently, investigators have shown that a regional but not global increase in $[K^+]_e$, can initiate sustained arrhythmias in a mechanism which is, in part, mediated by Ca^{2+} uncoupling from the

myofilaments and thereby, uncoordinated excitation-contraction coupling and non-uniform $[Ca^{2+}]_i$ (Miura *et al.*, 2012).

1.4.2.3 Ca^{2+}

During ischaemia cytosolic free Ca^{2+} $[Ca^{2+}]_i$ rises in a more delayed fashion than that observed for extracellular K^+ ion accumulation, although there is disagreement over the precise time-course of Ca^{2+} ion perturbation during ischaemia (Carmeliet, 1999).

The initial increase in cytosolic free Ca^{2+} ion concentration occurs as a consequence of the displacement of Ca^{2+} ions from their binding sites by accumulating H^+ (Carmeliet, 1999). The prolonged build-up of intracellular Ca^{2+} during ischaemia occurs secondary to Na^+ ion derangement. Reversal of the Na^+/Ca^{2+} exchanger occurs in a bid to reduce intracellular Na^+ levels and a direct outcome of this process is an intracellular Ca^{2+} ion overload. Coupled with this, uptake of Ca^{2+} ions by the sarcoplasmic reticulum (SR) is decreased. This reduced uptake by the SR is due to lowered V_{max} of the Ca^{2+}-ATPase, a consequence of both low ATP levels and oxygen free radical-induced disulphide bridge formation (Griese *et al.*, 1988; Kukreja *et al.*, 1991; Carmeliet, 1999; Ottolia *et al.*, 2013). Perturbations in $[Ca^{2+}]_i$ have also been shown to be caused by regional accumulation of $[K^+]_e$ following ischaemia (Miura *et al.*, 2012).

The ability of the cardiomyocyte to coordinate excitation and contraction is altered by perturbations of $[Ca^{2+}]$. For example, there is a reduction in conduction velocity and changes in prolonged refractoriness, which together can lead to the development of an environment which favours the generation of arrhythmias (Downar, Janse and Durrer, 1977). Accumulation of $[Ca^{2+}]_i$ in isolated myocytes have been shown to occur prior to the initiation of spontaneous fibrillation (Thandroyen *et al.*, 1991). In addition, Ca^{2+} overload in ischaemia has critical repercussions for the survival of those cells that have been reversibly damaged during ischaemia as contracture can develop and induce necrosis in potentially viable cells (Piper, Meuter and Schafer, 2003). The effects of ischaemia-induced elevations in $[Ca^{2+}]_i$ are not limited to ischaemia itself but can continue to affect cardiomyocytes post-reperfusion. More specifically, the uptake of Ca^{2+} by the mitochondria during ischaemia is a critical determinant of the likelihood of the opening of the mitochondrial permeability transition pore (mPTP), a large, non-specific conductance pore which spans the inner and outer mitochondrial membranes and whose opening facilitates mitochondrial membrane potential collapse and ultimately cell death (Crompton *et al.*, 1999; Hausenloy *et al.*, 2003; Hausenloy, Duchen and Yellon, 2003; Halestrap, Clarke and Javadov, 2004).

1.4.2.4 H^+

When the myocardium becomes ischaemic there is retention of CO_2 and an increase in proton generation, resulting in a net change in intracellular pH and acidosis (Carmeliet, 1999). The intracellular acidosis due to ischaemia results because of increased production of H^+ and deficient removal of these species. Intracellular H^+ levels are increased during ischaemia due to three main mechanisms; (1) ATP production shifts to the glycolytic pathway for the duration of ischaemia with concomitant H^+ generation, (2) ATP hydrolysis is coupled with the production of H^+ and (3) H^+ removal is deficient due to CO_2 retention (Watson *et al.*, 1984; Allen *et al.*, 1985; Eisner *et al.*, 1989; Dennis, Gevers and Opie, 1991; Carmeliet, 1999). Intracellular acidosis has been implicated as a factor that may contribute to K^+ accumulation in the extracellular space. Experiments have shown that alteration of pH in ischaemic myocardium modifies $[K^+]_e$ immediately (Kleber, Riegger and Janse, 1987; Janse and Wit, 1989). The electrophysiological consequences of acidosis of ischaemic myocardium are a fall in resting membrane potential, reduced upstroke velocity and a concurrent increase in action potential duration. These acute changes in the cardiac action potential of cells within the ischaemic zone are brought about because of altered ion channel function due to acidosis. Altered conduction and cardiac action potential retardation can increase the likelihood of the occurrence of triggered activity (Coraboeuf, Deroubaix and Coulombe, 1979; Carmeliet, 1999).

1.4.3 Cell necrosis

Occlusion of a coronary vessel induces ischaemia and irreversible cell death, or necrosis, of cardiomyocytes in the area at risk. The incidence of cellular necrosis post-occlusion is well correlated with infarct size. The cellular changes which result from ischaemia include alterations in mitochondrial structure and contractile machinery of the myocytes with eventual cellular oedema. Factors which affect the rate of progression of cellular necrosis upon reperfusion and the likelihood of its development include the levels of collateral flow and degree of oxygen consumption at the time of occlusion (Schaper and Schaper, 1988). Furthermore, the loss of plasma membrane integrity (LPMI), an event typical of necrotic cell death, has been shown to occur within 1 hour of reperfusion and lasts for up to 24 hours thereafter. Reactive oxygen species are thought to drive LPMI within the first 3 hours post-reperfusion, after which time the principal mediator is complement deposition, specifically of complement protein 3 (C3; Charlagorla *et al.*, 2013).

1.4.4 Functional alterations resulting from ischaemia

The most prominent functional alterations that transpire consequent to MI are arrhythmias. Arrhythmias develop within minutes of coronary occlusion and can precipitate fatality (Janse and Wit, 1989). The electrophysiological changes which ensue subsequent to a reduction in blood flow are the main impetus for initiation of arrhythmias (Janse and Kleber, 1981). Moreover, the prolongation of the cardiac action potential coupled with the increased $[Ca^{2+}]_i$ induces negative lusitropic effects, which concurrently increase left ventricular end diastolic pressure and cardiac stiffness (Bers, Eisner and Valdivia, 2003; Madonna, Cevik and Nasser, 2013).

1.4.5 Ischaemic preconditioning

When a period of ischaemia due to coronary occlusion is repeatedly interrupted by ischaemia and reperfusion which precede the prolonged occlusion, the infarct size is reduced by three quarters and in addition, the incidence of both ischaemia and reperfusion-induced arrhythmias are reduced (Vegh *et al*., 1990; Kaszala *et al*., 1996). Furthermore, after the initial occlusion event there is no additional ATP loss or necrosis (Jennings *et al*., 1978; Reimer and Jennings, 1979; Murry *et al*., 1986; Schott *et al*., 1990). This process is termed ischaemic preconditioning. The infarcts in preconditioned models are not laterally confluent like those of non-preconditioned models; in addition the infarct size in preconditioned animals is smaller regardless of the level of collateral flow (Murry *et al*., 1986). The mechanisms that induce infarct reduction in preconditioning are proposed to be slowing of ATP depletion in all occlusion episodes after the first and wash out of catabolites during each reperfusive window (Schott *et al*., 1990). With regard to the anti-arrhythmic effect of preconditioning, the cardioprotective effects of preconditioning were shown to be lost when the cyclo-oxygenase pathway is blocked (Vegh *et al*., 1990; Parratt and Vegh, 1994).

1.5 Electrophysiology of the ischaemic myocardium and arrhythmias

Arrhythmias can result from both ischaemia and reperfusion (Janse and Wit, 1989). Numerous factors are responsible for the occurrence of arrhythmias and these are termed substrate, trigger, and modulating factors. Substrate factors manifest as the physical consequences of ischaemic episodes, for example functional conduction block. Trigger factors are required to be present for arrhythmias to develop; they include ventricular premature depolarisations and tachycardia. The substrate and trigger factors can be altered by the modulating factors, for example electrolyte alterations (Janse and Wit,

1989). Together these factors define the risk of susceptibility of arrhythmia development (Shah *et al*., 2005).

Experimental coronary occlusion produces electrophysiological changes within minutes, the most noticeable change being depolarisation of cells in the ischaemic zone, together with changes in transmembrane ion balance (Janse and Wit, 1989). These electrophysiological changes bring about changes in cardiac action potential, including shortening and decreased amplitude (Janse and Kleber, 1981; Janse and Wit, 1989). Ultimately the outcome of sustained ischaemic events is an increase in the occurrence of arrhythmias. Following experimental coronary occlusion two periods of arrhythmias have been identified; those occurring within the first 2-10 minutes are termed immediate ventricular arrhythmias (IVA or phase Ia) and those occurring between 12 and 30 minutes are termed delayed ventricular arrhythmias (DVA or phase Ib) (Harris, 1950; Kaplinsky *et al*., 1979; Janse and Wit, 1989). These two phases are collectively termed Harris phase I arrhythmias and this bimodal appearance of arrhythmias has been identified in many species, including the rat and pig (Harris, 1950; Kaplinsky *et al*., 1979; Hirche *et al*., 1980; Parratt, 1982; Janse and Wit, 1989).

1.5.1 Altered cardiac action potentials

Along with depolarisation of cells within the ischaemic zone, alterations in action potential morphology occur when coronary occlusion is induced. Depolarisation immediately subsequent to occlusion perpetuates conditions that favour prolongation of action potential duration and extension of the effective refractory period of those cells within the ischaemic zone. These changes in action potential morphology are also well correlated with catecholamine flux and transient changes in $[K^+]_e$ and the late I_{Na}. These changes act negatively on conduction velocity (Waldo and Kaiser, 1973; Janse and Wit, 1989; Carmeliet, 1999; Noble and Noble, 2006; Madonna, Cevik and Nasser, 2013). Additional changes include a decrease in amplitude and upstroke velocity (Janse and Wit, 1989). These reductions in cardiac action potential parameters occur because the K^+ efflux induced by MI-stimulated opening of the K_{ATP} channels, this causes a small amount of membrane depolarisation and thereby a reduction in upstroke velocity (Wirth *et al*., 2000).

It is the non-homogenous topology of changes in the cardiac action potential in regional ischaemia that is the critical mediator in the generation of arrhythmias (Madonna, Cevik and Nasser, 2013). Cells in the border zone experience different alterations in ion balance compared with those of the normal myocardium and will thus exhibit different alterations in action potential morphology, thus causing dispersion in the degree of repolarisation of myocytes within the heart through which injury currents flow (Pollard *et al*., 2002). The inhomogeneity and pathological changes in action potentials provide a

suitable milieu for the formation of arrhythmias, for example early afterdepolarisations (EAD) and reentry (Carmeliet, 1999; Madonna, Cevik and Nasser, 2013).

1.5.2 Altered automaticity

Automatic depolarisation is a characteristic typical of the pacemaker cells of the SA and AV nodes. Altered automaticity has been implicated as a cause of ventricular arrhythmias (Mangoni and Nargeot, 2008). Spontaneous depolarisations in non-pacemaker cells increase in occurrence following a period of MI (Wit and Bigger, 1975). Moreover, in atrial myocytes which exhibit spontaneous depolarisation, inhibition if the late I_{Na} was found to reduce the frequency of such spontaneous activity (Song *et al*., 2008). Other tissues within the impulse-conducting system are capable of automaticity. In physiological conditions the more rapid depolarisation of the SA node prevents these alternative sites dominating and becoming the automatic focus of the heart. Ischaemia increases the rate of depolarisation of these alternative sites and thus increases the likelihood that they become the automatic focus (Carmeliet, 1999).

1.5.3 Triggered activity

This form of non-reentrant arrhythmia, as the name suggests, requires the presence of a trigger for its initiation. Triggered activity is characterised by both EADs and DADs (Janse and Wit, 1989; Carmeliet, 1999). These arrhythmias occur either before or after completion of repolarisation of an action potential and are induced by changes in $[Ca^{2+}]_i$ in the ischaemic cardiomyocyte (Song *et al*., 2008; Shryock *et al*., 2013).

EADs are depolarisations that occur during the plateau or repolarisation phase of the action potential. These derangements can result from slow repolarisation and subsequent reactivation of Na^+ and Ca^{2+} channels, combined with consequent Na^+-mediated Ca^{2+}-overload (Song *et al*., 2008). The persistent inward sodium current (late I_{Na}) which persists in the ischaemic myocardium is responsible for prolonging the action potential duration and thereby the induction of EADs (Hoey *et al*., 1994; Song *et al*., 2008; Hoyer *et al*., 2011; Shryock *et al*., 2013).

DADs are transitory depolarisations that appear immediately after repolarisation has completed. The conditions required for this form of arrhythmia are spontaneous Ca^{2+} release from the SR and low intracellular K^+, again conditions typical following a period of ischaemia (Luo and Rudy, 1994;

Carmeliet, 1999; Shryock *et al*., 2013). The oscillations in $[Ca^{2+}]_i$ driven by SR release activate a transient inward current (I_{TI}) which consequently facilitates the formation of DADs (Song *et al*., 2008).

1.5.4 Reentry

Reentry is characterised by a conduction disorder that results in the reexcitation of tissue by an impulse that would ordinarily have died out (Shryock *et al*., 2013). The conditions required for the formation of reentry are; (1) the presence of a functional unidirectional conduction block and (2) slow conduction coupled with short refractory periods (Quan and Rudy, 1990). These criteria are fulfilled during ischaemia (Carmeliet, 1999). During the initial 30 seconds following ischaemia there is a noticeable inhomogeneity between the action potential refractory periods, mainly due to spatial dispersion of excitability because of differing $[K^+]_e$ in myocytes of the ischaemic zone and those of the border (Wit and Janse, 2001; Sidorov *et al*., 2011). This dispersion of refractoriness is postulated to cause conduction delay and fractionation of wavefronts and this can lead to generation of arrhythmias by a reentrant mechanism (Levites, Banka and Helfant, 1975; Ferrier, Moffat and Lukas, 1985).

1.6 Reperfusion injury

Readmission of oxygen-rich blood to the myocardium subsequent to ischaemia is the most effective means of salvaging those cells which have undergone pathological changes but which still remain retrievable (Litt *et al*., 1989; Maxwell and Lip, 1997). This process, which is known as reperfusion, is itself a double-edged sword; reperfusion may rescue potentially viable cells but can result in additional endothelial destruction, lipid peroxidation, and arrhythmias in the form of oscillatory afterpotentials and ventricular tachycardia (Ferrier, Moffat and Lukas, 1985; Pogwizd and Corr, 1987; Maxwell and Lip, 1997). Reperfusion injury is defined as the death of cells which, although damaged after ischaemia, are potentially viable and die as a direct result of the events which occur at reperfusion (Kloner, Przyklenk, and Whittaker, 1989; Park and Lucchesi, 1999). Reperfusion injury has been identified as occurring both immediately succeeding reperfusion (less than 3 hours) and later (more than 3 hours), and different pathologies define each (Litt *et al*., 1989; Entman *et al*., 1991; Jordan *et al*., 1999). Furthermore, the pathogenesis of reperfusion has been demonstrated to be due to oxygen-derived free radicals and/or Ca^{2+} (Maxwell and Lip, 1997).

Mitochondria play a critical role in determining the viability of myocytes subject to ischaemia and reperfusion. On one hand, loss of the mitochondrial serine-threonine protein kinase, PTEN (phosphatase and tensin homologue on chromosome 10)-induced kinase 1 (PINK1)), can increase the likelihood of cell death following ischaemia by, in part, increasing opening of the mPTP, a large, non-specific pore found in the inner mitochondrial membrane (IMM), whose opening alone upon reperfusion of the ischaemic myocardium can induce cell death (Siddall *et al*., 2013). On the other hand, it has been shown that mitochondria may be mediators of ischaemic preconditioning (IPC) by sub-lethal opening of this very pore (Hausenloy *et al*., 2010).

1.6.1 Reperfusion-induced arrhythmias

The appearance of arrhythmias within seconds of reoxygenation of ischaemic myocytes was first confirmed by Tennant and Wiggers in 1935. Since then it has be noted that in reperfusion there is a high incidence of ventricular tachycardia (VT), ventricular fibrillation (VF) and derangement of the idioventricular rate (Levites, Banka and Helfant, 1975; Naimi *et al*., 1977; Penkoske, Sobel and Corr, 1978; Pogwizd and Corr, 1987). The mechanisms by which these reperfusion arrhythmias develop are thought to differ from those of ischaemic arrhythmias, since ischaemic arrhythmias are slow onset and reperfusion arrhythmias appear almost instantaneously (Kaplinsky *et al*., 1981). There is a correlation between the duration of the ischaemic period and the severity and incidence of arrhythmia occurrence post-reperfusion (Janse and Wit, 1989). The definitive mechanisms that are responsible for the onset and maintenance of reperfusion arrhythmias remains elusive, however, putative mechanisms include enhanced automaticity, depression of action potentials and inhomogeneity (Kaplinsky *et al*., 1981; Murdock *et al*., 1980; Pogwizd and Corr, 1987; Janse and Wit, 1989). Immediately after reperfusion has been initiated, VF dominates over VT (Janse and Wit, 1989). Following a period of ischaemia, action potentials may be depressed and some cells may even become inexcitable. Re-oxygenation aids the recovery of transmembrane action potentials however they still exhibit depressed amplitudes and upstroke velocities. This depression of action potential activity is not uniform however and there is acute inhomogeneity. This inhomogeneity combined with the intra- and extracellular accumulation of metabolites and ions increases the chance of reentry (Janse and Wit, 1989). A non-reentrant mechanism was suspected after it was demonstrated the reperfusion is associated with an increase in the idioventricular rate (Pogwizd and Corr, 1987). This change in the idioventricular rate enhances ventricular automaticity and thus the likelihood of arrhythmic activity, for example ventricular tachycardia (Kaplinsky *et al*., 1981). Finally, prolongation of refractory periods post-reperfusion has been shown to be coupled with increases in ventricular ectopic activity, such as EAD and DAD (Levites, Banka and Helfant, 1975). In this thesis, I have not studied reperfusion arrhythmias but instead focused on ischaemia-induced arrhythmias.

1.7 The pharmacology of cannabinoids

Cannabinoids have been used medicinally for almost 4000 years and are the natural bioactive ligands of the plant *Cannabis sativa* (Brown, 2007; Ryberg *et al*., 2007). This plant produces in excess of 80 cannabinoids and they principally act at two established cannabinoid receptors, namely the CB_1 and CB_2 receptors, however their activity has been detected at sites distinct from this including the GPR55 receptor, TRPV1 ion channel and 5-HT_3 receptors (Brown, 2007; Pertwee, 2007; Console-Bram, Marcu and Abood, 2012). The most abundant compounds derived from *Cannabis sativa* are (-)-Δ^9-tetrahydrocannabinol (Δ^9–THC) and cannabidiol (CBD) (Turner *et al*., 1980; Chen and Buck, 2000). Synthetic cannabinoid ligands have been produced in the hope of producing compounds that are selective for CB_1 and CB_2 receptors.

1.7.1 Receptors

In the early 1990s molecular cloning established the principal targets of cannabinoid action, they are the CB_1 and CB_2 receptors, however more recent studies have identified putative targets at sites distinct from them (Pertwee, 2006; Brown, 2007; Pertwee, 2007). Both the CB_1 and CB_2 receptors are G-protein coupled 7-transmembrane spanning receptors (GPCRs) that exhibit constitutive basal activity (Turner *et al*., 1980; Chen and Buck, 2000; Lepicier *et al*., 2006; Pertwee, 2006).

1.7.2 CB_1

The CB_1 receptors are chiefly expressed within neuronal tissues and in particular the nerve terminals of the central and peripheral nervous system, where they mediate the psychotropic effects of Δ^9-THC and inhibit the constitutive release of both excitatory and inhibitory neurotransmitters like acetylcholine and noradrenaline (Szabo and Schlicker, 2005; Pertwee, 2006; Brown, 2007; Ralevic and Kendall, 2009). In the central nervous system the density and location of CB_1 receptors is related to the sites at which cannabinoids generate their psychotropic effects (Devane *et al*., 1988; Pertwee, 1997).

In addition to those CB_1 receptors expressed in the central nervous system (CNS), CB_1 receptors have been shown to be present in the myocardium and densely populate the cytoplasmic and perinuclear area of cardiomyocytes. In addition, the hypotensive effects of cannabinoids are mediated by

activation of the CB_1 receptors which occupy sympathetic nerve terminals of the peripheral nervous system (Ishac *et al*., 1996; Pertwee, 1997; Weis *et al*., 2010).

CB_1 receptors together with the endocannabinoid ligands are thought to play an important modulatory role within the cardiovascular system. In particular, bolus administration of the endocannabinoid, AEA, has been shown to induce triphasic changes in cardiovascular parameters in anaesthetised rats and mice. More specifically, phase I is characterised by a significant but transient fall in blood pressure coupled with a reduction in cardiac contractility and a rise in total peripheral resistance (TPR). Phase II is typified by a brief pressor response and a rise in cardiac contractility. Finally, phase III produces a protracted fall in blood pressure together with a reduction in cardiac contractility, total peripheral resistance (TPR) and heart rate (Lake *et al*., 1997; Malinowska *et al*., 2001; Pacher *et al*., 2004). Two discrete receptors mediate these AEA-induced effects. Capsazepine and ruthenium red have been shown to block the AEA-mediated effects characteristic of phase I, indicative of involvement of the vanilloid TRPV1 receptors. The CB_1 antagonist, SR141617A, abolished the prolonged hypotension of phase III at doses effective at blocking CB_1 receptors (Lee and Lundberg, 1994; Malinowska *et al*., 2001; Szolcsanyi, 2000). The receptors responsible for phase II may include TRPV1 (Pacher *et al*., 2004); calcium channels (Kwolek *et al*., 2005) and a combination of N-methyl-D-aspartate (NMDA) receptors, β-adrenoceptors and thromboxane A_2 receptors (Malinowska *et al*., 2010). Other cannabinoids only activate part of the effects AEA has the capacity to induce (Malinowska, Baranowska-Kuczko and Schlicker, 2012).

CB_1 receptors are part of a family of GPCRs and are Class A rhodopsin α-group members (Fredriksson *et al*., 2003). The CB_1 receptor sequence is highly conserved between rats and humans with 93% and 97% identity at the nucleic and amino acids levels, respectively (McPartland and Glass, 2003; Console-Bram, Marcu and Abood, 2012). Coupling between the components of the CB_1 receptor and subsequent downstream signal cascades occurs principally through $G\alpha_{i/o}$ proteins positively to A-type outward and inwardly rectifying potassium channels and also to N- and P/Q-type calcium channels. This coupling to calcium channels may be responsible for the signal cascade accountable for mediation of the psychoactive effects of cannabinoids (Pertwee, 1997; Lepicier *et al*., 2006; Pertwee, 2006; Brown, 2007). CB_1 receptors can affect adenylate cyclase activity by one of two routes, negatively, if coupled to $G\alpha_{i/o}$ proteins or positively, if coupled to $G\alpha_s$ proteins. This indiscriminate CB_1 receptor coupling may be tissue specific and this may explain any tissue specific actions of cannabinoids (Pertwee, 2006).

Recent studies demonstrated that the CB_1 receptor displays constitutive activity (i.e. activation which occurs devoid of an agonist) after it was noted that mitogen activated protein kinase (MAPK) activity in CHO cells transfected with the CB_1 receptor showed basal MAPK activation levels higher than

those in untransfected cells. What is more, the CB_1 antagonist, SR141716A could reduce both agonist induced changes in MAPK activation and MAPK activation in cells expressing CB_1 that had not been treated with an agonist (Bouaboula *et al*., 1997; Console-Bram, Marcu and Abood, 2012). CB_1 receptors also possess at least one allosteric site, occupation of which can modulate positively or negatively the activation induced by a direct agonist (Price et al., 2005; Adam et al., 2007; Horswill et al., 2007; Navarro et al., 2009; Pertwee *et al*., 2010)

CB_1 receptors have been shown to exist as homomers and also heteromers and oligodimers with other classes of GPCRs; which may permit cross-talk between multiple receptors subtypes (Pertwee, 2006; Waldeck-Weiermair *et al*., 2008; Kargl *et al*., 2012). Furthermore, the biochemical properties of the receptors involved in these heteromers can be altered. More specifically, CB_1/GPR55 receptor heteromers show enhanced activity in terms of both ERK1/2 phophorylation and nuclear factor of activated T cell (NFAT) activation (Kargl *et al*., 2012) and those with the adenosine A2A receptor can influence the depressant effects of cannabinoids on motor function (Carriba *et al*., 2007).

1.7.3 CB_2

In 1993 Munro et al. identified a second type of cannabinoid receptor subsequently termed the CB_2 receptor. This receptor showed 44% sequence similarity to the CB_1 receptor (Pertwee, 1997). High affinity, saturable binding sites identified as the CB_2 receptor have been detected in inflammatory cells, cells of the haematopoietic system and in the central nervous system in pathophysiological settings which are associated with neuroinflammation (Pertwee, 1997; Brown, 2007).

It is widely accepted that CB_2 receptors are capable of modulating inflammatory cell migration and cytokine release. Furthermore evidence suggests that CB_2 knock-out mice can lack T-cell-activating responses to Δ^9–THC in subtypes of immune cells (Brown, 2007). CB_2 receptors are negatively coupled to adenylate cyclase and positively coupled to MAPK, unions which are mediated by the $G_{i/o}$ proteins (Pertwee, 2006; Pertwee *et al*., 2010).

1.7.4 GPR55

A number of the vasoactive effects of cannabinoids are not consistent with those which would be expected upon activation of the designated cannabinoid receptors, leading to the suggestion that additional cannabinoid receptors exist. More specifically, the synthetic cannabinoid, abnormal

cannabidiol (abn-CBD) has been shown to induce mesenteric vasodilatation and SR141716A-sensitive hypotension in both $CB_1^{-/-}$ and $CB_2^{-/-}$ mice. The haemodynamic changes mediated by abn-CBD are also susceptible to CBD-mediated inhibition (Jarai *et al*., 1999). Furthermore, the concentration of SR141716A, a typical CB_1 receptor antagonist, required to block the actions of AEA in the vasculature are higher than those required to competitively inhibit the CB_1 receptor (Hiley and Kaup, 2007). Recent studies have identified GPR55, an orphan GPCR, as a putative cannabinoid receptor (Brown, 2007). Functional fingerprint identification has however, documented that the human GPR55 receptor does not share a similar fingerprint to CB_1 or CB_2 receptors; this may infer that although this receptor may operate as a cannabinoid receptor it may not have a typical cannabinoid binding pocket. Moreover, GPR55 receptors in their active conformations have a binding pocket which is highly hydrophilic in contrast to the highly hydrophobic binding pocket that typifies the CB_1 and CB_2 receptors (Petitet, Donlan and Michel, 2006; Henstridge *et al*., 2011; Kotsikorou *et al*., 2011). GPR55 forms part of the purinergic receptor family and was first identified as a possible cannabinoid target in a patent from GSK that described activation of this receptor by AM251 and SR141617A in yeast host strains that expressed human GPR55 receptors (Brown, 2007).

Over recent years there has been much debate over the likelihood that GPR55 is a legitimate third cannabinoid receptor. Early studies demonstrated that GPR55 is activated by the AEA, 2-AG and CP55940 in human embryonic kidney 293 (HEK293) cells transfected with this receptor and this GPCR was initially purported to be the novel cannabinoid receptor responsible for the vasoactive effects of abn-CBD (Hiley and Kaup, 2007; Ryberg *et al*., 2007). Subsequent study expanded the ligand pharmacology to include O-1602 and AM251 (Table 1.2; Fischbach et al., 2007; Johns *et al*., 2007; Waldeck-Weiermair *et al*., 2007). However, Johns et al. (2007) cast doubt on the likelihood of GPR55 acting as the endothelial anandamide receptor (eAR) having shown that administration of abn-CBD in $GPR55^{-/-}$ mice resulted in a rapid hypotension akin to that in wild type (WT) controls. Furthermore, vasodilatation responses to both abn-CBD and O-1602 were of similar magnitude in mesenteric arteries taken from $GPR55^{-/-}$ and WT control mice. More recently the selectivity of some of the previously proposed ligands of GPR55 has been called into question in a study which identified only lyosphosphatidylinositol (LPI) as an agonist and discounting the activity of abn-CBD and O-1602 (Kapur *et al*., 2009). These data however are not supported by a subsequent study in which it was shown that both LPI and O-1602 can act as agonists at GPR55 to increase miniature excitatory postsynaptic currents (mESPCs) in CA1 pyramidal cells (Sylantyev *et al*., 2013). What is clear is that the precise ligand pharmacology of GPR55 remains elusive and, therefore, also the physiological and pathophysiological consequences of GPR55 activation. More recently a number of GPR55-selective, synthetic benzoylpiperazine compounds have come to the fore which lack activity at CB_1 and CB_2. Interestingly, unlike LPI, these compounds are not equipotent at rodent and human GPR55 but rather,

specifically activate the human receptor. This may suggest species-distinct binding domains within this receptor family (Brown *et al*., 2011; Henstridge *et al*., 2011).

Ryberg et al. (2007) were one of the first groups to attempt to identify the downstream signal transduction pathways induced by GPR55 activation. An antibody against the C terminus of $G\alpha_{13}$ had the capacity to inhibit GTPγS binding, an effect not reproducible with antibodies against $G\alpha_{i1/2}$, $G\alpha_{i3}$ or $G\alpha_{s}$. GPCRs shown to couple to G_{13} proteins have also been revealed as having other G proteins targets, those for GPR55 have yet to be identified (Brown, 2007). More recently, $G\alpha_{13}$ activation by GPR55 have been implicated in Ca^{2+} release from internal stores, transduction conferred by $G\alpha_{13}$-RhoA-ROCK-PLC with subsequent induction of inositol 1,4,5-triphosphate (IP_3) and thus Ca^{2+} release from the endoplasmic reticulum (ER) (Henstridge *et al*., 2009; Henstridge *et al*., 2011; Moreno-Navarrete *et al*., 2012). GPR55-induced intracellular Ca^{2+} mobilisation has been shown to induce a number of transcriptional factors for example, ERK 1/2 and p38 MAPK, whose activation may be involved in modulating cell physiology (Oka *et al*.,2007; Henstridge *et al*., 2010; Oka *et al*., 2010).

There is mounting evidence that GPR55 may play a significant role in physiological function and in some instances it may even promote progression of several pathologies. Early studies in $GPR55^{-/-}$ mice showed that following adjuvant-induced inflammation there was no inflammatory mechanical hyperalgesia for up to 14 days after adjuvant injection (Staton *et al*., 2008). This pointed to an important role for GPR55 in the treatment of neuropathic pain. It is thought that the presence of GPR55 on large diameter neurones is why they may play a role in inflammatory pain (Lauckner *et al*., 2008; Henstridge *et al*., 2011). As described earlier, some effects of cannabinoids in the vasculature cannot be rationalised as a CB_1 or CB_2 receptor-mediated response; the vasodilatation induced by Δ^9-THC cannot be inhibited by the selective CB_1 receptor antagonist, rimonabant (O'Sullivan, Kendall and Randall, 2005) moreover; abn-CBD can induce vasorelaxation in rat mesenteric artery from both $CB_1^{-/-}$ and $CB_2^{-/-}$ mice (Jarai *et al*., 1999). This cannabinoid ligand sensitive vascular receptor was purported to be GPR55, however, similar changes in mean arterial blood pressure were observed in WT and $GPR55^{-/-}$ mice, thus, the likelihood that this vascular receptor is GPR55 was contentious. In a more recent study, a fluorescent derivative of the reputed GPR55 receptor agonist, AM251, has identified GPR55 receptors in mouse mesenteric arteries (Daly *et al*., 2010). The role of GPR55 in regulating inflammatory cell migration and chemotaxis is controversial; that being said, mRNA for this receptor has been identified on human neutrophils among other inflammatory cell types (Balenga *et al*., 2011; Henstridge *et al*., 2011). GPR55 receptors have also been implicated in the control of cancer cell proliferation, more specifically, in cancers of the brain, pancreas and breast (Guzman, 2003; Sarfaraz *et al*., 2008; Henstridge *et al*., 2011; Caffarel *et al*., 2012).

1.7.5 Vanilloid receptors

Caterina et al. first cloned the vanilloid receptor in 1997. It is a ligand-gated, non-selective cation channel that has a membrane topology and binding domain configuration similar to typical cation channels (Malinowska, Kwolek and Gothert, 2001; Hogestatt and Zygmunt, 2002; Howlett *et al*., 2002). It belongs to a family of transient receptor cation channels which have a 6 transmembrane spanning domain and a non-selective cation channel (Pertwee *et al*., 2010). This channel has been acknowledged as participating in the mediation of inflammation-induced thermal hyperalgesia with ligands for this receptor including heat (>43°C), capsaicin and pH (Caterina *et al*., 2000; Davis *et al*., 2000; Hogestatt and Zygmunt, 2002). Although this channel was originally identified as the recognition site of capsaicin, studies have demonstrated that some cannabinoids (AEA, N-arachidonoyl dopamine (NADA), and CBD) can bind and act as full agonists at this site (Zygmunt et al., 1999; Smart et al., 2000; Bisogno *et al*., 2001; Molinowska, Kwolek and Gothert, 2001; Ross et al., 2001; Hogestatt and Zygmunt, 2002; Howlett *et al*., 2002; Pertwee *et al*., 2010). The site at which these cannabinoids interact is thought to be the same as its typical agonist, capsaicin (Jordt and Julius, 2004). Moreover, CB_1 receptors can inhibit vanilloid receptor function by TRPV1 receptor dephosphorylation, the consequences of which may influence the pro-inflammatory signaling cascade induced by TRPV1 (Yang *et al*., 2013). TRPV1 receptors and CB_1 receptor activation by cannabinoids has also been shown to reduce carcinoma invasion (Farsandaj, Ghahremani, and Ostad, 2012). This again highlights the potential significance of cannabinoid receptors and their ligands which activate both receptors and moreover, their roles in physiological and pathological processes.

1.7.6 Allosteric sites

Literature exists which has described functional allosteric modulation of 5-HT_3 receptors by cannabinoids, furthermore, a putative allosteric site has been identified on the CB_1 receptor itself (Barann *et al*., 2002; Godlewski *et al*., 2003; Price *et al*., 2005; Ross, 2007). An allosteric binding site is topographically discrete from the typically recognised orthosteric binding site and binding of modulators to such a site causes a conformational change in the receptor which facilitates modification of binding affinity and efficacy of ligands for the orthosteric site (Price *et al*., 2005; Ross, 2007).

Cannabinoids can exert effects discrete from those of a psychotropic nature, including modulation of emesis and sensations of pain; this is a function shared with 5-HT_3 receptors. It has previously been acknowledged that 5-HT_3 receptors possess sites whose properties permit allosteric modulation. Barann et al. (2002), were the first to confirm that cannabinoid receptor ligands inhibit 5-HT_{3A}-

induced current in transfected HEK293 cells. This modulation of 5-HT_{3A}-induced current was proposed to occur as a result of allosteric modulation, since radioligand binding studies confirmed absence of the typical cannabinoid receptors in this process. Further study confirmed the presence of cannabinoid agonist-induced modulation of 5-HT_3 receptors *in vivo* (Godlewski, Gothert and Molinowska, 2003).

In 2005 Price et al. (2005) identified novel allosteric modulators of the CB_1 receptor. Three compounds, ORG27569, ORG27759 and PSNCBAM-1 have been recognised as having ligand-dependent effects, typical of allosteric modulators, in HEK293 cells expressing hCB_1 receptors. These compounds were shown to bind to the allosteric site and induce a conformation change such that the agonist affinity for the orthosteric site was increased. This postulated allosteric site on CB_1 receptors in thought to be recognised by small synthetic molecules (Price *et al.*, 2005; Ross, 2007). More recently, it has been shown that ORG27569 and PSNCBAM-1 increase the effect of the orthosteric agonist by increasing the rate at which the CB_1 receptor is desensitised and delaying its internalisation, while the early signalling events driven by CB_1 at $G\alpha_i$ are unaffected (Cawston *et al.*, 2013).

Cannabinoid agonists, both synthetic and endogenous, have been shown to modulate ion channel function in an allosteric or non-competitive manner (Pertwee *et al.*, 2010). Derivatives of arachadonic acid were shown to modulate the 1,4-dihydropyridine (DHP) calcium channel antagonist binding site in a manner suggesting that these derivatives were non-competitive antagonists (Johnson *et al.*, 1993). A more recent investigation found that AEA blocked the DHP binding site, in addition to 1,5-benzothiazepine and phenylalkylamine sites on the LTCC (Shimasue *et al.*, 1996). These binding sites are acknowledged to interact by way of allosteric modulators and thus, these authors suggested that AEA might be acting at one of these sites and that this interaction allosterically modulates the binding and/or function of the other sites. Potassium (K^+) channels have also been shown to be sensitive to cannabinoids in a CB_1 receptor-independent mechanism and at nanomolar to low micromolar concentrations (Pertwee *et al.*, 2010). AEA can inhibit K^+ conductance by suppressing the delayed rectifier outward current of the voltage gated K^+ channel (Vignali *et al.*, 2009).

Voltage-gated sodium channels are no less affected by allosteric modulation by cannabinoids, namely, 2-AG and NADA. These cannabinoids inhibit binding of [(3)H]batrachotoxinin A-20alpha-benzoate ([(3)H]BTX-B) to site 2 of the sodium channel in a non-CB_1 dependent, allosteric manner (Duan, *et al.*, 2008; Duan, Zheng and Nicholson, 2008).

1.7.7 Ligands

The agonists of the classical cannabinoid receptors include those which are synthetic or natural ligands of *Cannabis sativa* and those that are endogenous cannabinoids. Stereoselectivity for each of the receptors occurs; furthermore, these ligands are capable of acting at sites distinct from the CB_1 and CB_2 receptors. Development of selective cannabinoid receptor antagonists rapidly followed the discovery of the cannabinoid binding sites. Pertinent examples include AM251, SR141617A and AM630 (Pertwee, 1997; Howlett *et al*., 2002; Pertwee *et al*., 2010).

1.7.8 Agonists

The agonists (Table 1.1) of the classical cannabinoid receptors are typically classified according to their chemical structure, for example;

The classical cannabinoids – A category that includes dibenzopyran analogues and derivatives e.g. Δ^9-THC and CBD.
The non-classical cannabinoids – A category comprising of bi- and tri-cyclic analogues of Δ^9-THC, for example CP 55,940 and DALN. This group was crucial in finding functional cannabinoid receptors within the brain.
The aminoalkylindole cannabinoids – Typified by WIN-55,212-2, and JWH-015.
The eicosanoid cannabinoids – The endocannabinoids are all eicosanoids and pertinent examples include N-arachidonylethanolamide, also known as AEA, and 2-arachidonyl glycerol (2-AG). The eicosanoids are susceptible to hydrolysis by fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL) (Johnson and Melvin, 1986; Pacheco *et al*., 1991; Martin *et al*., 1991; Devane *et al*., 1992; Pertwee, 1997; Howlett *et al*., 2002; Pertwee, 2006).

The identification of the CB_1 receptor subsequently led to the discovery of an endogenous ligand active at the CB_1 receptor, namely, AEA (Di Marzo *et al*., 1998). The endocannabinoid system (ECS) describes the principal cannabinoid receptors and their endogenous ligands, the endocannabinoids. Endocannabinoid synthesis and subsequent release is initiated by post-synaptic increases in intracellular calcium (Pertwee, 2007). Some of the cannabinoid receptor agonists are capable of marked stereoselectivity for either receptor; this is principally due to the presence of a chiral centre within the compound. However, CB_1 and CB_2 selective agonists have been developed, examples being arachidonyl-2'-chloroethylamide (ACEA) and L-759633 respectively. CB_1 selective agonists are chiefly AEA analogues and CB_2 selective agonists Δ^9-THC analogues (Pertwee, 1997; Howlett *et al*.,

2002). The authentication of reputed cannabinoid receptor agonists is reliant upon *in vivo* and *in vitro* bioassays such as the mouse tetrad for CB_1 receptor agonists and membrane transfection experiments for CB_1 and CB_2 receptors agonists (Pertwee, 1997).

Cannabinoid	Receptor	Experiment	Properties	Pharmacological target	Species	Reference
(-)-Δ⁹-THC	CB_1	*In vitro* displacement of [^{3}H]CP55,940	K_i 35.3 (nM)	Partial agonist of CB_1 and CB_2 receptors	Rat	Rinaldi-Carmona *et al*. (1994) Pertwee (2008)
	CB_2		K_i 3.9 (nM)			
SR141716A	CB_1	Hypothermia (*in vivo* antagonism of WIN55212-2)	ED_{50} i.p 0.11 mg kg^{-1}	Antagonist at CB_1	Rat	As above
CBD	CB_1	GTPγS binding (antagonism of EC_{50} of CP55,940)	IC_{50} >30000 (nM)	Partial agonist at CB_1 and CB_2 receptors	Rat	Ryberg *et al*. (2007) Thomas *et al*. (2007)
	CB_2					
AM251	CB_1	GTPγS binding (antagonism of EC_{50} of CP55,940)	IC_{50} 8 (nM)	Antagonist at CB_1	Rat	Ryberg *et al*. (2007)
	CB_2		IC_{50} 2915 (nM)			
ACEA	CB_1	Radioligand binding assay	K_i 1.4 (nM)	Agonist at CB_1	Rat	Hillard *et al*. (1999)
ACEA	CB_2	Radioligand binding assay	K_i 3.1 (μM)	Agonist at CB_1	Rat	Hillard *et al*. (1999)

Table 1.1 The pharmacology of selected cannabinoids at the CB_1 and CB_2 receptors.

Cannabinoid	Receptor	Experiment	Properties	Pharmacological target	Species	Reference
CBD	GPR55	GTPγS binding (antagonism of EC_{50} of CP55,940)	IC_{50} 445 (nM)	Antagonist at GPR55	Rat	Ryberg *et al.* (2007)
AM251	GPR55	GTPγS binding (antagonism of EC_{50} of CP55,940)		Agonist at GPR55	Rat	
O-1602	GPR55	GTPγS binding	EC_{50} 1.4 (nM)	Agonist at GPR55	HEK293 cells expressing GPR55	Johns *et al.* (2007)

Table 1.2 Pharmacology of selected cannabinoids at the GPR55.

1.7.9 Antagonists

Some notable competitive, surmountable antagonists of the cannabinoid receptors have been identified. Among those are SR141716A, WIN 56,098, AM251 and AM630, of which some have receptor selectivity (Pertwee, 1997; Howlett *et al*., 2002; Pertwee, 2006).

The first cannabinoid receptor antagonist to be identified was WIN 56,098 after it was shown that this compound antagonises WIN 55212-2-induced inhibition of cAMP generation. Subsequently, SR141716A demonstrated [^{3}H] CP 55,940 displacement from cannabinoid receptors; furthermore this compound was shown to inhibit typical cannabinoid-induced effects (Pacheco *et al*., 1992; Rinaldi-Carmona *et al*., 1994; Pertwee, 1997). Curiously however, later publications reported that SR141716A produced effects opposite to those expected of a cannabinoid agonist at CB_1 receptors, a response characteristic of an inverse agonist. It was further proposed that the CB_1 receptor must exist in a constitutively active state, capable of downstream signal transduction despite the absence of an agonist and that SR141716A must bind and induce the receptor to assume a constitutively inactive state (Milligan *et al*., 1995; Compton *et al*., 1996; Pertwee, 1997; Pertwee, 2005; Pertwee, 2006). However, studies in models of mutant CB_1 receptor and CB_1 transfected endocannabinoid-free Chinese hamster ovary (CHO) cells, have shown that some of the inverse agonist effects of SR141716A occur independently of endocannabinoid activation of the receptor (Bouaboula *et al*., 1997; Pan *et al*., 1998; Pertwee, 2005).

Selective cannabinoid receptor antagonists which block agonist induced activation of CB_1 or CB_2 receptor have been developed and include AM251, the CB_1 receptor selective antagonist and AM630, the CB_2 receptor antagonist. Antagonists of cannabinoid receptors have been exploited in the clinic for the treatment of obesity, such as rimonabant (Despres, Golay and Sjostrom, 2005; Van Gaal *et al*., 2005; Pertwee, 2006). Other selective antagonists of the CB_1 receptor include, AM251 and AM281, whose use has also highlighted the potential for inverse agonism at the CB_1 receptor. AM251 can induce depression of food intake in rats (McLaughlin et al., 2003; Pertwee, 2005) and AM281 can increase locomotor activity in mice (Cosenza *et al*., 2000; Pertwee, 2005). It is likely that some of the inverse agonist activities of these compounds are due to antagonism of the effects of endocannabinoid induced activation of CB_1 receptors.

1.8 Cannabidiol

CBD was first isolated in the early 1940's yet its structure was not fully described until the early 1960's (Mechoulam, Parker and Gallily, 2002). This constituent of the plant *Cannabis Sativa* is non-psychotropic and most abundant only after Δ^9–THC and, like Δ^9–THC, CBD is a classical cannabinoid (Pertwee, 1997, Pertwee, 2006). The structure of CBD is depicted in figure 1.3:

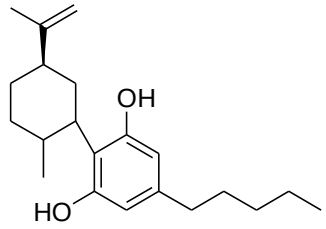

Figure 1.3 The chemical structure of the phytocannabinoid (-)-cannabidiol. Adapted from Pertwee., (2007).

Like many other cannabinoids CBD is a chiral molecule, the (-) (3R, 4R) enantiomer occurs naturally in *Cannabis sativa*, whilst the (+) (3S, 4S) enantiomer is a synthetic derivative. The chirality of CBD can affect receptor activity (Hanus *et al*., 2005; Pertwee, 2004). In [^{3}H]CP55940 displacement experiments CBD was shown to displace at concentrations in the micromolar range, suggesting low affinity for the classical cannabinoid receptors (Showalter *et al*., 1996; Thomas *et al*., 2004; Pertwee, 2007; Thomas *et al*., 2007). Indeed, CBD has been shown to antagonise CP55940-induced inhibition of electrically evoked contractions of vas deferens however, this antagonism occurred at concentrations below those required for established CB_1 receptor activity (Pertwee *et al*., 1995; Pertwee *et al*., 2002; Thomas *et al*., 2004). It was concurrently recognised that CBD's activity at the CB_1 receptor was similar to that observed for the CB_1 -selective antagonist, SR141716A (rimonabant). Furthermore, like rimonabant, a CB_1 inverse agonist/antagonist, CBD exhibits an ability to increase electrically evoked contractions in mouse vas deferens (Pertwee *et al*., 1995; Pertwee *et al*., 2002; Thomas *et al*., 2004; Thomas *et al*., 2007). Therefore, despite brief confusion regarding whether CBD could indeed act at classical cannabinoid receptors, evidence to date infers that CBD can act as a non-competitive antagonist of CB_1 receptors. What's more it has also been proposed that any inverse agonist activity exhibited by CBD is CB_1 receptor-mediated following experiments which showed no inhibition of [^{35}S]GTPγS binding in untransfected CHO cells membranes compared with hCB_1-CHO cell membranes (Pertwee, 2007; Thomas *et al*., 2007). With regard to CB_2 receptors, Thomas et al., (2007) identified that in hCB_2-CHO cell membranes CBD had a K_i of 4.9µM for the displacement of [.

^{3}H]CP55940, furthermore CBD demonstrated inhibition of [^{35}S]GTPγS binding in these membranes similar to that of the CB_2 receptor inverse agonist, SR1445283.

Further putative sites of action for CBD have been identified and include; the post-synaptic α_1-adrenoceptor (at which allosteric modulation by CBD is proposed), a possible site of the dopamine D_2 receptor and the AEA membrane transporter, which results in modulation of endocannabinoid uptake (Bloom, 1984; Bloom and Hillard, 1985; Howlett *et al*., 2002; Pertwee *et al*., 2002; Pertwee, 2004). The activity of CBD at GPR55 has also been investigated and it was identified that although it does not affect GPR55 GTPγS binding and thus is probably not an agonist, it has the capacity to antagonise that action of CP55940 in the same assay. CBD was also shown to block the activation of rhoA, rac1 and cdc42 in GPR55-expressing HEK293 cells stimulated with AEA or O-1602. Thereby, these data suggest a role for CBD as an antagonist at GPR55 (Ryberg *et al*., 2007). Finally, CBD has been shown to modulate the transcriptional activity of peroxisome proliferator-activated receptor gamma (PPARγ; O'Sullivan *et al*., 2006).

CBD is capable of initiating a plethora of actions some of which may confer cardioprotective effects, for example:

- Inhibition of delayed rectifier K^+ channels in isolated guinea-pig ventricular myocytes. Derangement of potassium channel function has been implicated in the pathophysiology of MI-reperfusion injury (Carmeliet, 1999; Mamas and Terrar, 1998).

- Further modulation of ion channel function includes that of the cardiac LTCCs, which in the presence of 0.32 to 3.2μM CBD, have demonstrated reduced Ca^{2+} movement (Mamas and Terrar, 1998). Conversely, another group have demonstrated that CBD facilitated elevations in $[Ca^{2+}]_i$ in hippocampal cells in a partly LTCC-dependent manner (Drysdale *et al*., 2006; Pertwee *et al*., 2010). Ca^{2+} overload is noted to play a significant role in the perturbation of normal electrophysiological properties of cardiac myocytes during MI-reperfusion injury (Lubbe, Podzuweit and Opie, 1992; Ugdyzhekova *et al*., 2001).

- In *in vivo* models of stroke CBD was shown to reduce infarct volume, which is thought to be due principally to the ability of CBD to alter inflammatory cell migration. Functional inverse agonist activity at the CB_2 receptor is acknowledged to accompany retarded immune cell migration to sites of inflammation (Chen and Buck, 2000; Hampson *et al*., 2000; Pertwee, 2004; Pertwee, 2007).

- It has been found that in addition to modulating immune cell function, CBD is capable of modifying production and/or release of mediators of immune cell function, for example cyclooxygenase, lipooxygenase and inflammatory cytokines like TNF-α. (White and Tansik, 1980; Furmukong *et al*., 1991; Watzl, Scuderi and Watson, 1991a; Watzl, Scuderi and Watson, 1991b; Srivastava, Srivastava and Brouhard, 1998; Costa *et al.*, 2002; Pertwee, 2004). In a viral model of multiple sclerosis, CBD was shown to protect from inflammation by reducing expression of vascular cell adhesion molecule (VCAM) expression and cytokine pro-interleukin-IL-1β (IL-1β) (Mecha *et al*., 2013). VCAM expression is significantly up-regulated following acute MI in rats (Grieve *et al*., 2013). Moreover, Il-1β expression also increases after induction of MI in rats (Sandanger *et al*., 2013). Thus, the cardioprotective effects of CBD could be in part attributed to an anti-inflammatory action.

- CBD is a potent anti-oxidant, as determined by cyclic voltammetry and through analysis of a spectrophotometric assay of oxidation in a Fenton reaction. Moreover, CBD has been shown to offer comparable protection with butylated hydroxytoluene (BHT) in a model of glutamate neuronal toxicity (Hampson *et al.*, 2000; Booz *et al.*, 2011). Generation of ROS occurs during MI and it is thought that the accumulation of ROS and opening of the mPTP upon reperfusion may be key protagonists in reperfusion-induced myocardial injury (Halestrap, Clarke and Khaliulin, 2007; Santos *et al.*, 2011).

- The endogenous autocoid adenosine plays a significant cardioprotective role during both ischaemia and reperfusion by impeding development of necrosis and moderating endothelial cell injury, effects mediated by A_1, A_2 or A_3 receptors (Olafsson *et al*., 1987; Babbitt, Virmani and Forman, 1989; Pitarys *et al*., 1991; Zhao *et al*., 1993; Jordan *et al*., 1997). Durst et al. (2007) postulated that the cardioprotective effect of CBD observed after left anterior descending (LAD) coronary artery ligation was conferred by the adenosine receptors. Adenosine accumulates in the ischaemic myocardium and it has been suggested that its cardioprotective actions are the product of inhibition of neutrophil migration in to the ischaemic and necrotic zones, inhibition of platelet aggregation and inhibition of ATP-sensitive K^+ channels (Cronstein *et al*., 1983; Cronstein *et al*., 1989; Engler et *al*., 1986; Kitakaze *et al*., 1991; Gross and Auchampach, 1992; Toombs *et al*., 1992; Zhao *et al*., 1993). CBD has been shown to transactivate adenosine receptors and thus enhance adenosine signalling which could be cardioprotective (Wainwright and Parratt, 1993; Mecha *et al*., 2013).

- CBD has been established as being a modulator in endocannabinoid signalling. CBD can inhibit both hydrolysis and uptake of the endocannabinoid, AEA, which has been shown to induce a triphasic change in cardiovascular parameters (Bisogno *et al*., 2001; Malinowska, Kwolek and Gothert, 2001; Ross, 2003).

- CBD has been demonstrated to protect against serum-deprived cell death at submicromolar concentrations. This action is thought to be brought about by antioxidant-based mechanisms (Chen and Buck, 2000), which would prove beneficial following the burst of oxygen free radicals that occurs immediately upon reperfusion (Kloner, Przyklenk, and Whittaker, 1989).

Durst et al. (2007) demonstrated that CBD can reduce infarct size and also ameliorate reduction in left ventricular function following the induction of experimental occlusion. They noted that the reduction in infarct size was accompanied by a reduction in inflammatory cell migration to the area at risk. Furthermore, studies from our laboratory have demonstrated that acute administration of CBD is significantly anti-arrhythmic when given prior to the induction of coronary occlusion (Walsh *et al*., 2010). In a model of diabetic cardiomyopathy in mice, Rajesh et al. (2010) showed that chronic CBD treatment has the capacity to reduce cardiac dysfunction, fibrosis, ROS formation and NF-KB activation. Most recently, it was shown that CBD can ameliorate cardiotoxicity induced by doxorubicin (Fouad *et al*., 2013). This cardioprotection is thought to be afforded by reducing inflammatory mediators like TNFα, preventing the reduction of vital components of cardiac antioxidant reserves like selenium and zinc, and finally by stabilising $[Ca^{2+}]_i$. Moreover, CBD has been shown to be safe during chronic use and it has no detrimental effects on cardiovascular parameters (Bergamaschi *et al*., 2011; Martin-Santos *et al*., 2012; Fouad *et al*., 2013). Moreover, it can induce vasorelaxation in pre-constricted human arterial segments (Stanley and O'Sullivan, submitted for publication, see Stanley *et al*., 2013). Although there is evidence to suggest a cardioprotective role for CBD, there remains no firm consensus as to which receptors CBD may confer its effects through. It may be possible that the cardioprotective effects of CBD may be owed to the antagonism of CB_1 receptors and thereby, preferential activation of infarct sparing CB_2 receptors by upregulated endocannabinoids (whose synthesis and degradation is modulated such that concentrations are increased; Hajrasouliha *et al*., 2008; Kourliouros *et al*., 2009; Lim *et al*., 2009; Barana *et al*., 2010).

1.9 The endocannabinoid system and the cardiovascular system

As discussed earlier in this chapter (sections 1.7.2 and 1.7.3), CB_1 and CB_2 receptors are expressed in healthy myocardium (Weis *et al*., 2010). Recently, discussion regarding the modulation of the ECS as

a potential target for the pathologies of the cardiovascular system has increased (Batkai and Pacher, 2009; Montecucco and Di Marzo, 2012; Tuma and Steffens, 2012; Stanley *et al*., 2012; Pacher and Kunos, 2013; Pertwee, 2013). Functional ablation of the ECS in healthy animals produces little functional effect and therefore suggests that the ECS contributes little to normal physiological function (Pacher, Batkai and Kunos, 2006; Pacher and Mechoulam, 2011; Pacher and Kunos, 2013), moreover, the ECS produces endocannabinoids in response to pathophysiological stimuli. Initiators of such synthesis include; oxidative stress and inflammation, conditions apparent during episodes of tachyarrhythmias. Interestingly, neuronal tissues which contain excess $[Ca^{2+}]_i$ and are highly stimulated, are also characterised by upregulated endocannabinoid synthesis, an environment classically perpetuated during instances of atrial fibrillation (Kourliouros *et al*., 2009; Barana *et al*., 2010). The haemodynamic effects of cannabinoids are less apparent in conscious animals except in conditions of hypertension (Gardiner *et al*., 2002; Batkai *et al*., 2004; Wheal *et al*., 2007). This would again suggest that in pathological conditions the ECS may play an important role in cardiovascular function. Endocannabinoid function with respect to hypotension can have significant implications in the pathogenesis of some cardiovascular conditions, for example, the AEA generated by macrophages and the 2-AG generated by platelets in rats suffering from shock can induce hypotension when administered to healthy rats. Thus, suggesting that the endocannabinoids produced by these cells can contribute to shock-induced hypotension (Wagner *et al*., 1999). Given the potential role that endocannabinoids play in the exacerbation of cardiovascular pathologies it is important to give due consideration to their investigation of their involvement in such conditions and also the effects on normal physiological function. Moreover, even in healthy animals, the activation of CB_1 receptors induces negative ionotropy (Pacher *et al*., 2008). Agonism of the CB_2 receptor by HU-210 has been shown to reduce the area of necrosis and confers anti-arrhythmic effects following experimentally-induced ischaemia (Krylatov *et al*., 2001; Ugdyzhekova *et al*., 2001; Ugdyzhekova *et al*., 2002; Durst *et al*., 2007). In preclinical studies and in clinical trials, the selective antagonism or inverse agonism of CB_1 receptors improved cardiovascular risk factors in obese individuals (Despres, Golay and Sjostrom, 2005; Van Gaal *et al*,. 2005; Scheen *et al*., 2006; Nissen *et al*., 2008; Rosenstock *et al*., 2008; Despres *et al*., 2009; Hollander *et al*., 2010; Pacher and Kunos, 2013). Thus, both of the cannabinoid receptors have been shown to play a role in the modulation of cardiovascular function in health and disease.

1.10 Statement of aims

It is clear that the diverse pharmacology of CBD may grant some cardioprotective effects, however as yet the clear mechanisms of these reputed benefits are not established. Thus, the aim of this project is to further characterise the cardioprotective mechanisms of CBD.

The specific objectives of this project were to:

(a) Better understand the pharmacology of CBD by investigating the effect of bolus CBD administration on haemodynamic variables *in vivo* by pharmacological manipulation of the CB_1 and GPR55 receptors;

(b) Investigate and identify the receptors involved in the anti-arrhythmic effects of CBD in acute MI;

(c) Determine whether the cardioprotective effects of CBD are mediated by modulation of $[Ca^{2+}]_i$ at the level of the cardiomyocyte.

2. General Methods

2.1 In vivo studies

All *in vivo* studies were carried out in accordance with the Animal (Scientific Procedures) Act 1986 under personal licence number 60/1116. Animals were maintained to standards set out in the Home Office Code of Practice for the Housing and Care of Animals used in Scientific Procedures (Part 1, Section 2). They were housed in a specific pathogen-free facility, in conventional open caging, with a 12 hour light/dark cycle and had access to CRM pelleted diet (SDS diets, Essex, UK) and water *ad libitum*. Animals were accommodated in groups not exceeding 4 animals per cage and their environment enriched with corn cob, paper wool and a play tunnel. The temperature range in which they were accommodated was 19-22°C and humidity of 55 ± 10%. Animals were obtained on a daily basis from the Medical Research Facility (Aberdeen University) and transferred to the short-term holding facility within the Robert Gordon University. Animals did not undergo any experimental procedure until a 30 minute acclimatisation period had elapsed.

2.2 Surgical procedure

Animals were anaesthetised via intraperitoneal injection of sodium pentobarbital salt (60mg kg^{-1}), with maintenance of anaesthesia via bolus intravenous injection (3-4mg kg^{-1}). The necessity for administration of maintenance anaesthesia was determined by assessment of the pedal withdrawal reflex, with return of this reflex indicative of requirement for anaesthetic top-up. The assessment of the animal's reflex responses was carried out every 15-30 minutes. The left carotid artery and right jugular vein were isolated by blunt dissection using forceps and cannulated with portex polythene tubing (0.58mm ID x 0.96mm OD: Smiths Medical International Ltd., Kent, UK) to permit measurement of arterial blood pressure and drug administration, respectively. The arterial cannula contained heparised saline (NaCl; 40U ml^{-1}) and the venous cannula, saline (NaCl). A tracheostomy was also performed to allow artificial respiration on room air (54 strokes min^{-1}, volume 1.5ml $100g^{-1}$) in order to maintain blood pCO_2, pO_2 and pH within physiological limits (18-24mmHg; 100-130mmHg and 7.4, respectively; Harvard small animal respiration pump, Harvard Apparatus Ltd, Kent, UK). Mean arterial blood pressure (MABP) was recorded from the left carotid artery using a physiological pressure transducer (MLT844 physiological pressure transducer; ADIntruments, Oxfordshire, UK) and calculated using the following equation:

$$MABP = Diastolic\ BP + {}^{1}/_{3}\,(Systolic\ BP - Diastolic\ BP)$$

Equation 2.1 Equation for the calculation of MABP

Heart rate (HR) was calculated from a lead I ECG recorded from subcutaneous limb electrodes. Both MABP and HR data were relayed to the LabChart software (ADInstruments, Oxfordshire, UK) from the transducer via a bridge amplifier and Animal Bio Amplifier (ADIntruments, Oxfordshire, UK). Body temperature was monitored by a digital rectal thermometer and maintained at 37-38°C by way of a Vetcare heat pad (Harvard Apparatus Ltd, Kent, UK).

2.2.1 Surgical procedure for experimental coronary artery occlusion

Following completion of the basic surgery described in section 2.1, animals were prepared for *in vivo* experimental coronary artery occlusion (CAO), as previously described by Clark et al. (1980). In brief, the thorax was opened by performing a left thoracotomy between the third and fourth intercostal spaces, 2mm from the border of the sternum. The incision was then extended by cutting the third and fourth ribs, before the pericardium was removed by way of passing blunt dissection forceps carefully around the borders of the heart. The heart was then gently excised from the pericardium and externalised from the chest cavity, by application of gentle pressure to the right side of the chest and pushing the left side of the chest under the heart by means of forceps. A reverse cutting needle attached to silk suture (6/0, 11mm, 3/8c reverse cutting needle, Ethicon, Edinburgh, UK) was then passed around the left coronary artery, 3-4mm distally from its egress from the aorta, at the point distal to its emergence from the left atrial appendage. Subsequent to occlusion of the ligature, the heart was replaced in the chest cavity and the animal was allowed 25 minutes to stabilise before commencement of the experimental protocol. Ischaemia was induced by placing the suture ends in a round-ended plastic stopper, pulled firm so that the stopper made contact with the surface of the myocardium and secured in place using an augmented pipette tip, leading to occlusion of the coronary artery (Figure 3.1). Regional ischaemia was induced for 30 minutes; successful ligation of the coronary artery was confirmed by an increase in amplitude of the ECG, specifically the QRS complex, coupled with a fall in MABP. Any animal that experienced dysrhythmia or a MABP of less than 70 mmHg during the stabilisation period was excluded from further study. Cardiovascular variables were monitored throughout the protocol. Following completion of the experimental protocol, animals were terminated by anaesthetic overdose of sodium pentobarbital salt.

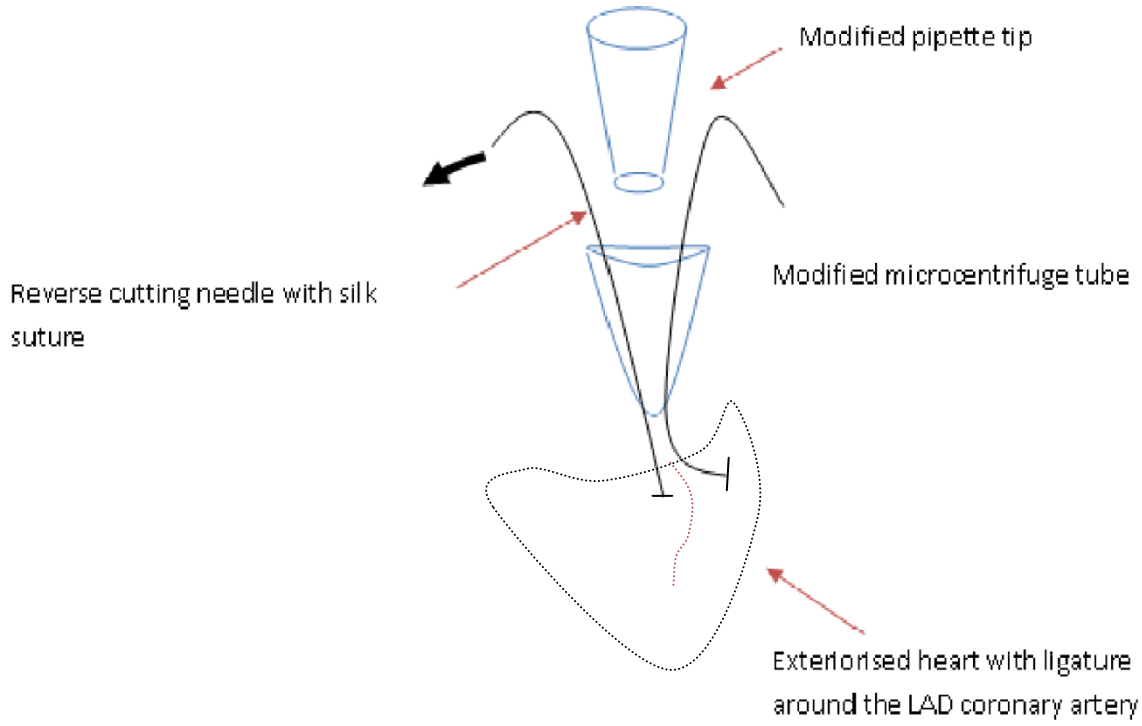

Figure 2.1 Occlusion device schematic

2.2.2 Analysis of haemodynamic responses

Haemodynamic responses to both occlusion and bolus drug administration were recorded using LabChart software, the data being relayed to this by way of a pressure transducer connected to an arterial cannula. To fully quantify both the magnitude and duration of the blood pressure changes, area under the curve analysis was performed on the 120 seconds pre- and 300 seconds post-bolus drug administration.

2.2.3 Analysis of ventricular arrhythmias

Ventricular arrhythmias were analysed according to the Lambeth Conventions (II): guidelines for the study of animal and human ventricular and supraventricular arrhythmias (Walker *et al*., 2013). A ventricular arrhythmia was defined as being a ventricular premature beat (VPB) which appears as an isolated premature QRS complex devoid of the accompanying P wave. A salvo referred to two (couplet) or three (triplet) consecutive VPB's. A succession of four or more VPB's, was defined as VT. The presence of VT was also associated with a slight fall in MABP (the BP trace remained pulsatile) and a HR which exceeded 500 beats min^{-1}. The occurrence of VF was defined as a lack of definable QRS complexes which tended to be associated with a precipitous fall in MABP to 0 mmHg. Arrhythmias were quantified as single VPB's, salvos, VT, total VPB's and total percentage incidence of both reversible and irreversible VF (r.VF and ir.VF, respectively). The distribution of arrhythmias occurring over the entire ischaemic period was also quantified. The incidence of arrhythmias that occurred over the entire ischaemic period was recorded.

2.3 Histological staining

2.3.1 Tissue processing

Following removal from the animal, whole heart tissues were fixed in ready-to-use 10% formalin (Shandon Formal Fixx) for 1-2 days, after which the hearts were sliced from base to apex in 2-3 mm slices and stored in phosphate buffered saline (PBS) at room temperature. Heart tissue samples were processed in cassettes through a sequence of graded alcohols, histosolve and paraffin wax using an autoprocessor (Citadel 1000, Thermo Shandon, Cheshire, UK) which employed the following steps:

Tissue processing protocol

1	Absolute alcohol	(100%)	2 hours
2	Absolute alcohol	(100%)	2 hours
3	Absolute alcohol/Histosolve (50:50)		1 hour
4	Histosolve (100%)		1 hour
5	Paraffin wax		2 hours
6	Paraffin wax		2 hours

Table 2.1 Tissue processing protocol employed for processing of tissue samples

Upon completion of tissue processing, heart tissues were embedded in paraffin wax using a tissue embedding centre (Histocentre 2, Thermo Shandon, Cheshire, UK). 4-5 µm sections were cut from blocks using a microtome (Finesse 325, Thermo Shandon, Cheshire, UK). Sections were floated out in a tissue floatation bath at 40˚C. These sections were then mounted on superfrost glass slides (for standard histology; Thermo Scientific, Leicestershire, UK) or polysine adhesion slides (for immunohistochemistry; Thermo Scientific, Leicestershire, UK). Drying was completed in a section dryer at 70˚C for 2-3 hours. Sections were mounted on slides at least 24 hours prior to commencement of histological analysis.

2.3.2 Immunohistochemical staining for GPR55

Detection of the presence and locality of GPR55 staining by immunohistochemistry (IHC) in myocardial tissue sections was completed by colleagues at AstraZeneca in Molndal, Sweden.

In brief, sections were cut (3-4 µm) from wax-embedded myocardial blocks, prepared as detailed in section 2.3.1, before being mounted on SuperFrost Ultra Plus adhesion slides (Thermo Scientific, Germany). Drying was completed at 60°C for 30-60 minutes. The IHC protocol described in the subsequent paragraph was completed in the automated IntelliPATH Flex Immunostainer (Biocare Medical, Concord, USA). Antigen unmasking was completed with the aid of a pressure cooker and DIVA decloaker and was completed in 5 minutes, followed washing with TBS automation wash buffer (TBS buffer). Non-specific antigen binding by the primary antibody was prevented using Peroxidazed 1, which quenched endogenous peroxidise activity, for 5 minutes, followed by the universal blocking reagent, Background Sniper, for 10 minutes. Each of these steps was succeeded by a wash in tris buffered saline (TBS) buffer solution. Following completion of the blocking steps, the slides were subject to incubation with the primary rabbit polyclonal antibodies directed to GPR55 (LS-

A162 and LS-A6817) for 1 hour. Once incubation with the primary antibody was complete, slides were washed in TBS buffer before continuing with primary antibody detection using the MACH 3 pre-prepared, biotin-free, micro-polymer detection kit; the first step using a horseradish peroxidise (HRP) probe lasted 10 minutes, with subsequent incubation with a HRP polymer for 10 minutes, each incubation with the detection kit called for TBS buffer washes to be completed twice. The chromogen used was Betazoid DAB chromogen kit and incubation with this agent persisted for 5 minutes, followed by rinsing in distilled water. Counterstaining was completed by addition of Tachas haematoxylin for 5 minutes. Following counterstaining, sections were dehydrated and cleared as described in

Table 2.2, before being permanently mounted in Mountex Mounting Medium and viewed at between x5 and x40 magnification. In these studies, positive controls were employed and in this instance, pancreatic tissue samples used. The negative control involved substituition of the MACH detection system with buffer. This aided in identifying any non-specific secondary antibody binding to tissue samples.

Dehydration and clearing protocol for GPR55 IHC

1	80% Alcohol	2 min
2	95% Alcohol	2 min
3	99.5% Alcohol	2 min
4	99.5% Alcohol	2 min
5	Xylene	5 min
6	Xylene	5 min

Table 2.2 Dehydration and clearing protocol employed by AstraZeneca in IHC for GPR55

2.4 Cardiomyocyte isolation

2.4.1 Preparation of cell extracts from adult rat ventricles

Those animals from which tissues were to be harvested for either histological analysis or isolation of cardiomyocytes, were euthananised under Schedule 1 by CO_2 asphyxiation, followed by cervical dislocation. The hearts were rapidly excised and the aorta cannulated before being mounted on the self-contained Langendorff apparatus (ML176 V; ADInstruments, Oxford, UK). The method used for enzymatic dispersion was an adaptation of a method described by Bates and Gurney, (1999). In brief,

retrograde perfusion at 37°C and a rate of 12ml min^{-1} was initiated with a nominally Ca^{2+}-free buffer solution (
Table 2.3).

Nominally Ca^{2+}-free buffer

	NaCl	120mM
	KCl	5.4mM
	$MgSO_4$	5mM
	Na-Pyruvate	5mM
	Glucose	20mM
	Taurine	20mM
	HEPES	10mM
	Distilled water	1000ml
	pH	6.96

Table 2.3 Composition of nominally Ca^{2+}-free buffer solution for cardiomyocyte isolation

This ensured that any residual blood was washed from the coronary vasculature, allowing the subsequent, enzyme-containing solutions, to fully perfuse the tissues via the coronary circulation. After 6 minutes, the buffer solution was changed to nominally Ca^{2+}-free buffer solution containing proteinase (4u ml^{-1}; Type XXIV), for 3 minutes, followed by perfusion with a nominally Ca^{2+}-free buffer solution containing collagenase (0.3mg ml^{-1}; Type II from *Clostridium histolyticum*) and hyaluronidase (0.6mg ml^{-1}; Type I-S) for 11 minutes. The hearts were then removed from the Langendorff apparatus, the atria removed and discarded and the ventricles incubated twice in collagenase (0.3mg ml^{-1}; Type II) at 37°C for 10 minutes. The tissue was then washed in Krebs-Henseleit buffer solution to prevent continued digestion and finely minced with a scalpel and stored at 37°C in Krebs-Henseleit buffer solution until required for loading with fluorescent dye (
Table 2.4). All buffer solutions used in the preparation of cell extracts were oxygenated with 95% O_2/5% CO_2.

Krebs-Henseleit buffer solution

NaCl	120mM
KCl	2.5mM
$MgCl_2$	2.5mM
NaH_2PO_4	0.5mM
KH_2PO_4	0.5mM
$NaHCO_3$	15mM
$CaCl_2$	1mM
Glucose	10mM
HEPES	5mM
Distilled water	1000ml
pH	7.4

Table 2.4 Krebs buffer solution for cardiomyocyte isolation

2.4.2 Detection of intracellular Ca^{2+} in isolated ventricular myocytes

Intracellular Ca^{2+} was detected by observing changes in fluorescence intensity in cells loaded with the Ca^{2+}-sensitive, cell-permeant, non-fluorescent acetoxymethyl (AM) ester of fluo-4. Once inside the cell, non-specific esterases cleave the lipophilic groups, which aid passage into the cell, yielding the green fluorescent, Ca^{2+} indicator, fluo-4. A small sample of minced ventricular tissue was placed in a 1.5ml micro-centrifuge tube containing 1ml of Krebs-Henseleit buffer solution and subjected to gentle mechanical separation by trituration with a glass-fired pipette. The cell extracts were then incubated with fluo-4 AM (5µM) for 30 minutes at room temperature (20 ± 3 °C). The fluorescence-labelled cell suspension (100µl) was then pipetted onto a cell perfusion chamber (capacity ~1ml) and mounted on the stage of a Leica DMI 4000B (Leica Microsystems, Milton Keynes, UK), before being washed with 1ml of Krebs buffer. Cells were first brought into focus using phase light and since fluo-4 exhibits a spectral wavelength of excitation maxima of 494nm and emission maxima of 516 nm when Ca^{2+} is bound, cells were illuminated using the red-green-blue (RGB) filter. Dye loading between different

cell isolates showed slight variability, as such, conditions for optimal image capture (aperture size, exposure etc) were set at the beginning of each experiment and maintained for the duration. Digital adjustment of the fluoresced cells was made on each isolate to ensure the ideal digital output.

2.5 Statistical analysis

All results are presented as mean ± SEM, unless otherwise stated. N indicates the number of animals used and n indicates the number of cells, each is detailed in the methods section of each chapter. Statistical significance ($P<0.05$) was determined by T-tests, ANOVA and Fisher's exact test as appropriate and the details are provide in the relevant methods section of each chapter.

2.6 Materials

For a detailed list of suppliers and preparation methods for each chemical, refer to appendix I.

All of the agents used in the *in vivo* studies were sourced from Sigma-Aldrich (Gillingham, UK), with the exception of heparin from Leo Laboratories (Buckinghamshire, UK) and finally, Immu-mount from Anatomical Pathology International (Cheshire, UK).

All chemicals for the completion of histological and immunohistochemical analyses were purchased from Biocare Medical (Concord, USA), with the exception of; PBS, TTC and Haematoxylin, Gill no.3 from Sigma-Aldrich (Gillingham, UK), normal horse blocking serum, ImmPRESS reagent, rabbit IgG, VectaMount and Vector VIP from Vector Laboratories (Burlingame, USA), Formal Fixx and toluidine blue from Thermo Fisher Scientific (Leicestershire, UK), CB_1 receptor primary antibody and AKT1 primary antibody from Abcam (Cambridge, UK), DIVA Decloaker and pertex mounting medium from HistoLab products (Gothernburg, Sweden), and finally, GPR55 primary antibody from MBL International Corporation (Massachusetts, USA).

All of the agents used in the isolation and study of cardiomyocytes were from Sigma-Aldrich (Gillingham, UK), with the exception of fluo-4 AM which was sourced from Molecular Probes Invitrogen (Paisley, UK).
All salts used in the preparation of buffer solutions were obtained from Sigma-Aldrich (Gillingham, UK), with the exception of NaCl and $MgCl_2$ from Fisher Scientific (Loughborough, UK), tri-sodium

citrate and glucose from Fisons (Loughborough, UK), trypsin from Invitrogen (Paisley, UK), $NaHCO_3$, Na-pyruvate and KOH from BDH Chemicals (Poole, UK).

3. Characterisation of the anaesthetised rat model of myocardial ischaemia

3.1 Introduction

The induction of experimental MI was first reported in 1862 (Bezold and Boyemann, 1862) and since then advances in the technique, be it *in vivo* or *ex vivo*, have produced highly reproducible models. Many of these models are regarded as being realistic representatives of clinical MI and have been routinely used to investigate novel cardioprotective agents (Johnston, MacLeod and Walker, 1983; as reviewed by Mitsos *et al*., 2009). The parameters commonly quantified in the rat model of acute MI include changes in heart rate and blood pressure, size of infarcted tissue and finally, the incidence and development of ischaemia-induced ventricular ectopic activity.

As discussed in Chapter 1, the distribution of arrhythmias over a 30 minute period of ischaemia in animal models is acknowledged to be bimodal and, although in close temporal association, both are defined by distinct phases (Clark *et al.*, 1980). Those arrhythmias which occur in the first 10 minutes after ligation of a coronary artery are termed phase Ia (or immediate ventricular arrhythmias) arrhythmias. During phase Ia the subepicardial zones in the ischaemic myocardium have been demonstrated to exhibit significant diastolic bridging, indicated by fractionated ECGs and likely due to non-homogenous conduction, which indicates a re-entry-based mechanism of induction (Scherlag *et al*., 1970; Boineau and Cox, 1973; Waldo and Kaiser, 1973; El Sherif, Scherlag and Lazzara, 1975; Kaplinsky *et al*., 1979; Russell *et al*., 1984; Carmeliet, 1999). In contrast, the arrhythmias that characterise phase Ib (delayed ventricular arrhythmias) are devoid of the slowed conduction within epicardial tissues and the heterogeneity that typifies phase Ia (Kaplinsky *et al*., 1979; Russell *et al*., 1984; Carmeliet, 1999). Phase Ib arrhythmias emerge between 12 and 30 minutes after cessation of local regional circulation, a time when action potentials are recovering but when ischaemic tissues experience significantly higher plasma levels of catecholamines. This phenomenon is induced by transmembrane efflux of catecholamines via the reversal of carrier-mediated transport (Schomig *et al*., 1984; Carlsson, Abrahamsson, and Almgren, 1985; Schomig *et al*., 1987; Schomig *et al*., 1988; Schomig *et al*., 1991).

3.2 Aim

The aim of the experiments described herein was to perform a series of control experiments using the anaesthetised rat CAO model, in order to characterise the effect of acute ischaemia on haemodynamics and the development of ventricular ectopic activity. This data was then used to identify the most important variables for subsequent drug intervention experiments detailed in Chapter 5.

3.3 Methods

3.3.1 Surgical procedure for experimental coronary artery occlusion

Male Sprague Dawley rats (250-450g; n=11) were prepared for surgery and experimental coronary artery occlusion as described in sections 2.2 and 2.2.1.

3.3.2 Assessment of haemodynamic parameters and occurrence of ventricular premature beats (VPBs)

Haemodynamic parameters and occurrence of VPBs were monitored throughout each experimental protocol, as described in sections 2.2.2 and 2.2.3.

3.3.3 Exclusion Criteria

Any animal that displayed a starting MABP of less than 70mmHg or those animals in which spontaneous arrhythmias developed in the stabilisation period were discounted from analysis. Furthermore, any animal which experienced irreversible VF were excluded from arrhythmia quantification.

3.3.4 Experimental Protocol

Figure 3.1 illustrates the experimental protocol used in this study. All control animals were allowed a 25 minute stabilisation period, which followed the completion of surgery but was before induction of coronary artery occlusion and was achieved by compressing the occlusion device against the surface of the myocardium (Figure 2.1). Coronary occlusion was maintained for 30 minutes, at which point the animals were euthanised by anaesthetic overdose. The exclusion criteria used in this study are defined in 3.3.3.

3.3.5 Statistical analyses

For the haemodynamic data, a one way ANOVA with Dunnett's post hoc test was used to compare pre- and post-ligation MABP and HR to determine the effect of ligation of the LAD coronary artery.

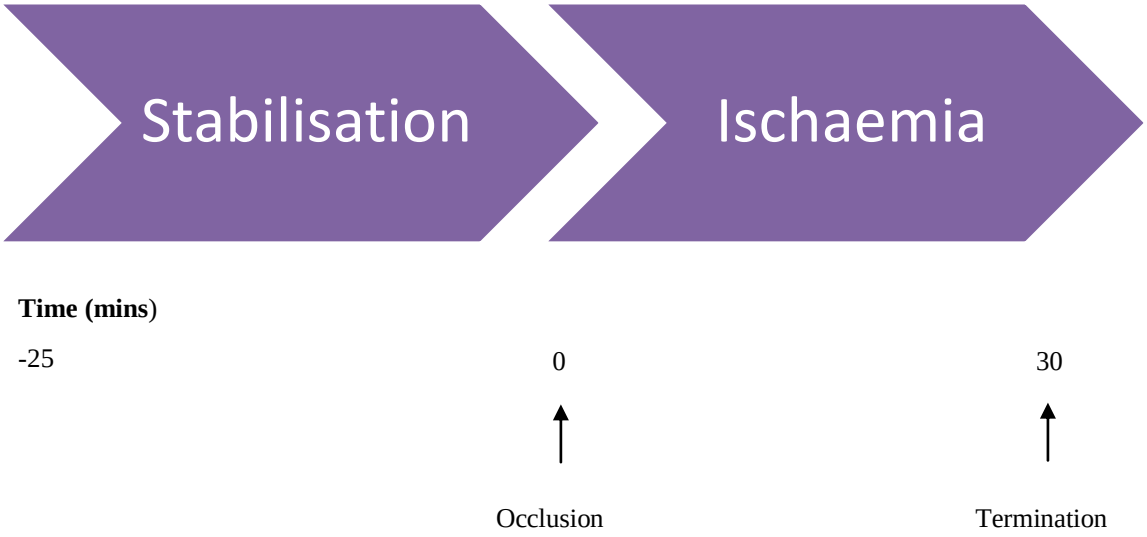

Figure 3.1 Experimental protocol for the characterisation of the haemodynamic and electrocardiographic response to CAO in anaesthetised rats.

3.4 Results

16 animals underwent the surgical procedure for experimental coronary occlusion. Of these, 5 died prior to or during the stabilisation period either as a result of excessive post-surgery bleeding or premature arrhythmias and were considered technical exclusions. Of the remaining 11 animals, 3 succumbed to post-ligation, ischaemia-induced, irreversible VF and were identified as arrhythmia-associated mortalities. The remaining 8 animals survived the entire period of coronary occlusion and the resultant ischaemia-induced ventricular arrhythmias were analysed.

3.4.1 Haemodynamic and electrocardiographic responses to coronary artery occlusion

The ligation of the coronary artery resulted in an instantaneous reduction in MABP with an accompanying increase in the amplitude of QRS complexes as evidenced by changes in R-wave height and ST segment elevation. Examples of these changes in haemodynamics are shown in Figure 3.2 and Figure 3.3. Induction of coronary artery occlusion resulted in a fall in MABP of approximately 55%, from 143 ± 8 mmHg (at the end of stabilisation) to 64 ± 12 mmHg (1 minute post-occlusion; $P<0.0001$; Figure 3.4). Blood pressure showed only a partial recovery to pre-ligation values during the occlusion period. In contrast, coronary occlusion had little effect on HR (442 ± 19 at the end of stabilisation vs. 427 ± 16 BPM at 1 minute post-occlusion; Figure 3.5).

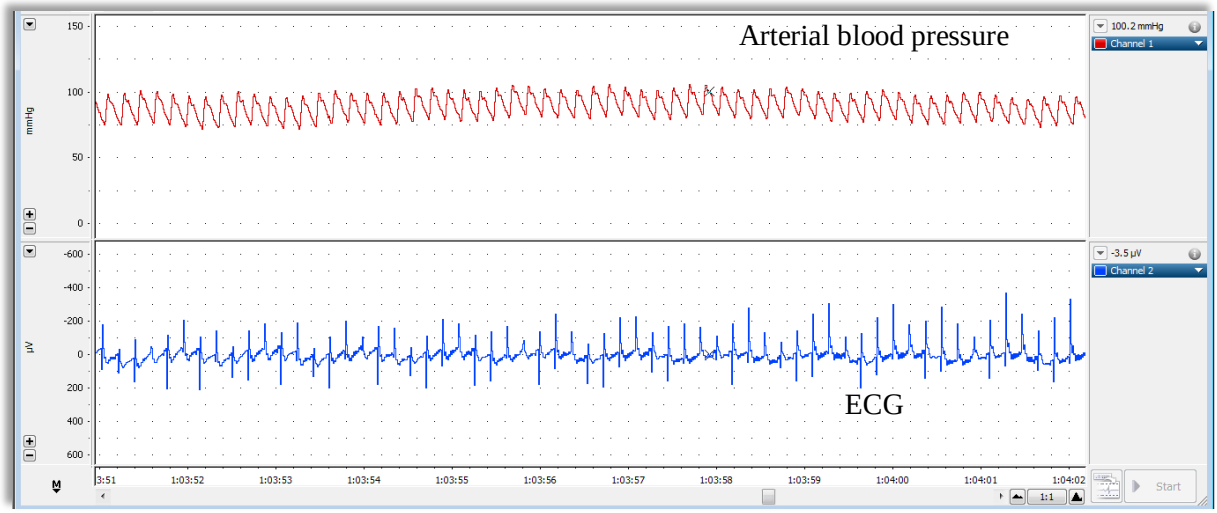

Figure 3.2 Baseline MABP and ECG trace. A typical example of a trace of arterial blood pressure (top panel) and ECG (bottom panel) in an anaesthetised rat.

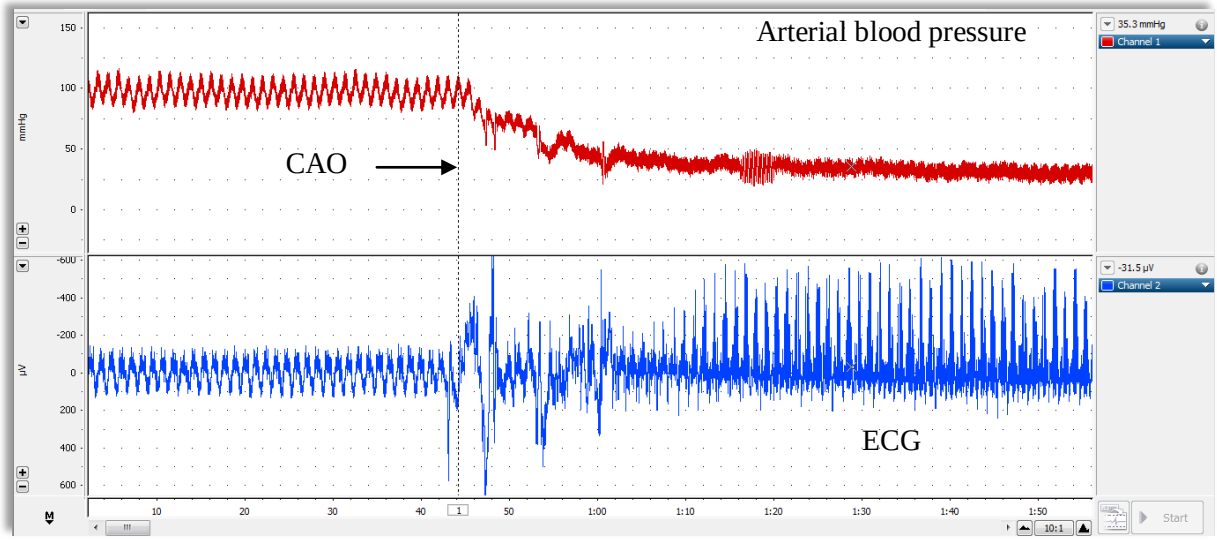

Figure 3.3 Ischaemia-induced changes in MABP and ECG. A typical trace example of changes in MABP and ECG observed on ligation of the coronary artery in an anaesthetised rat (indicated by the dashed line). There is a fall in MABP and an increase in the amplitude of the R wave of the ECG immediately upon CAO.

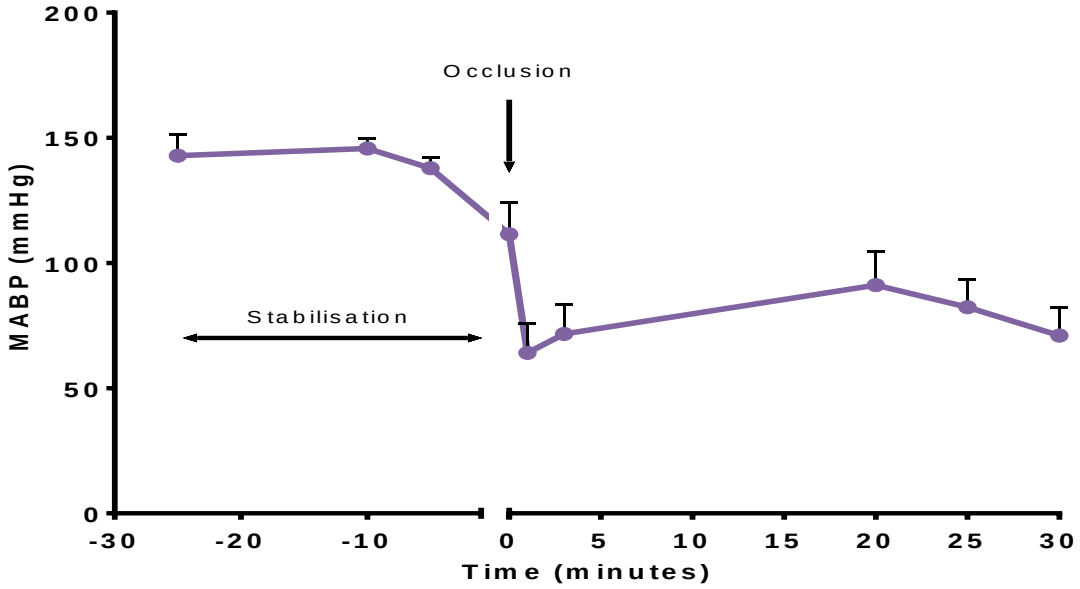

Figure 3.4 The impact of CAO on MABP. MABP was recorded throughout the surgical protocol . Data is expressed at mean ± SEM (n=8).

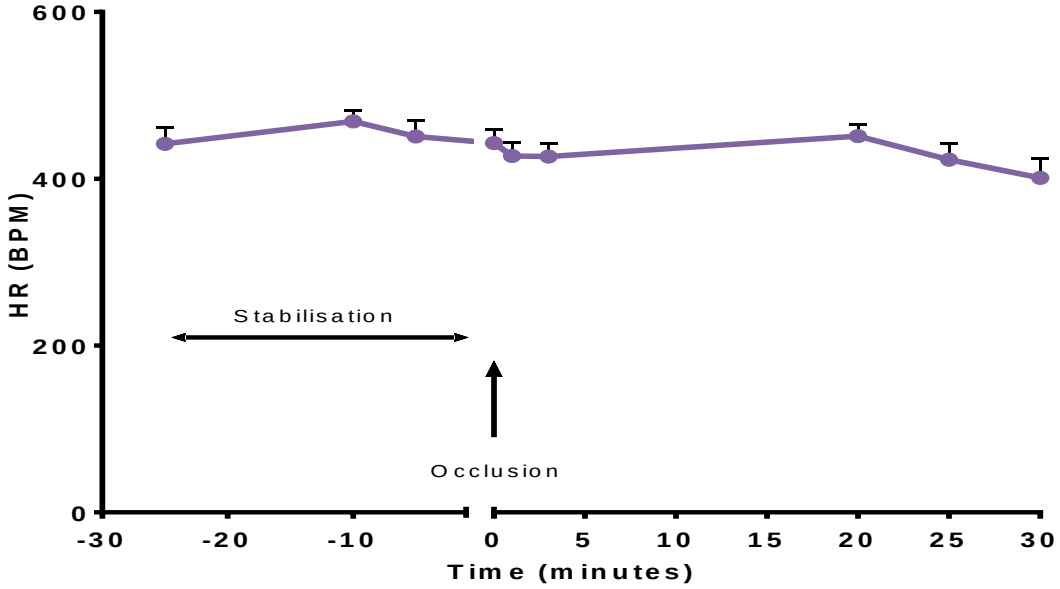

Figure 3.5 The impact of CAO on HR. HR was recorded throughout the surgical protocol. Data is expressed at mean ± SEM (n=8).

3.4.2 Characterisation of ischaemia-induced ventricular arrhythmias

Ligation of the coronary artery resulted in the development of ectopic activity within 3 minutes of occlusion. VPBs were defined as isolated, premature QRS complexes, devoid of an associated P wave and classified according to the Lambeth Conventions (Walker *et al*., 2013). Activity was categorised into single VPBs (Figure 3.6), salvos (couplets and triplets; Figure 3.7), VT (Figure 3.8) and VF (Figure 3.9).

3.4.3 Quantification of ischaemia-induced arrhythmias and VF incidence

Ventricular ectopic activity during a 30 minute period of ischaemia was composed of the VPBs defined above. However, the vast proportion of this activity occurred in the form of VT (Figure 3.10). Of those animals that underwent ligation of the coronary artery, 63% experienced reversible VF, and 27% died from irreversible VF. All of those animals in which irreversible VF was observed had experienced a preceding reversible VF event. Total incidence of VF for this group was 73% (Figure 3.11). The majority of ventricular ectopic activity occurred in the 7-20 minute post-occlusion period, with peak ectopic activity occurring 9 minutes post-ligation (242 ± 69 VPBs; Figure 3.12). Furthermore, the majority of arrhythmias presenting as single VPBs and salvos occurred during the Phase Ia period of (0-10 minutes post-occlusion) ischaemia-induced ventricular arrhythmias (979 vs. 788 VPBs; Phase Ia vs. Phase Ib, respectively).

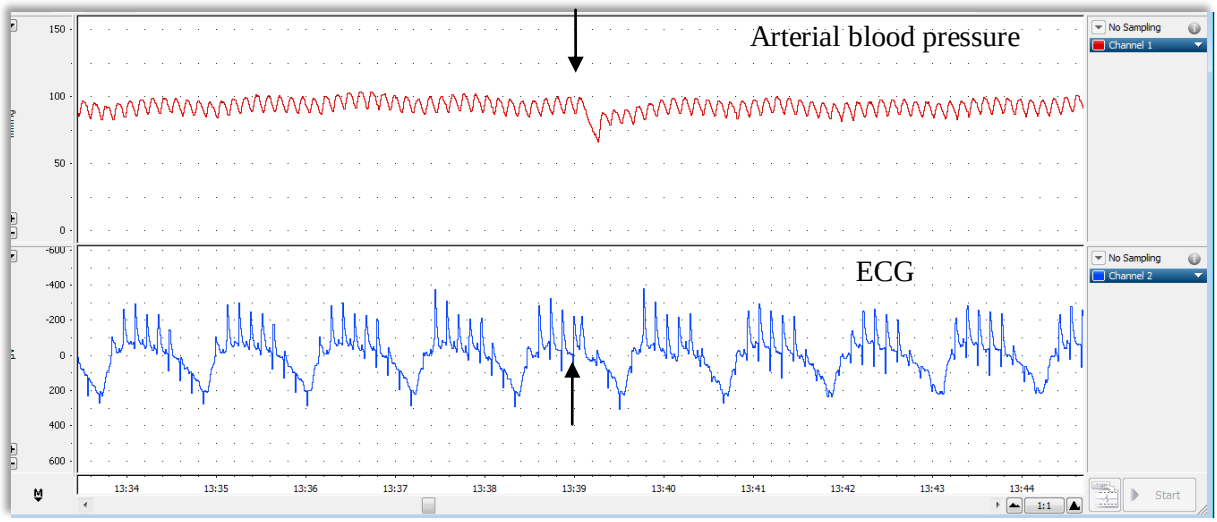

Figure 3.6 Ventricular ectopic beat (VPB). A typical trace of a series of single VPBs, as indicated by the arrows, with associated fall in MABP.

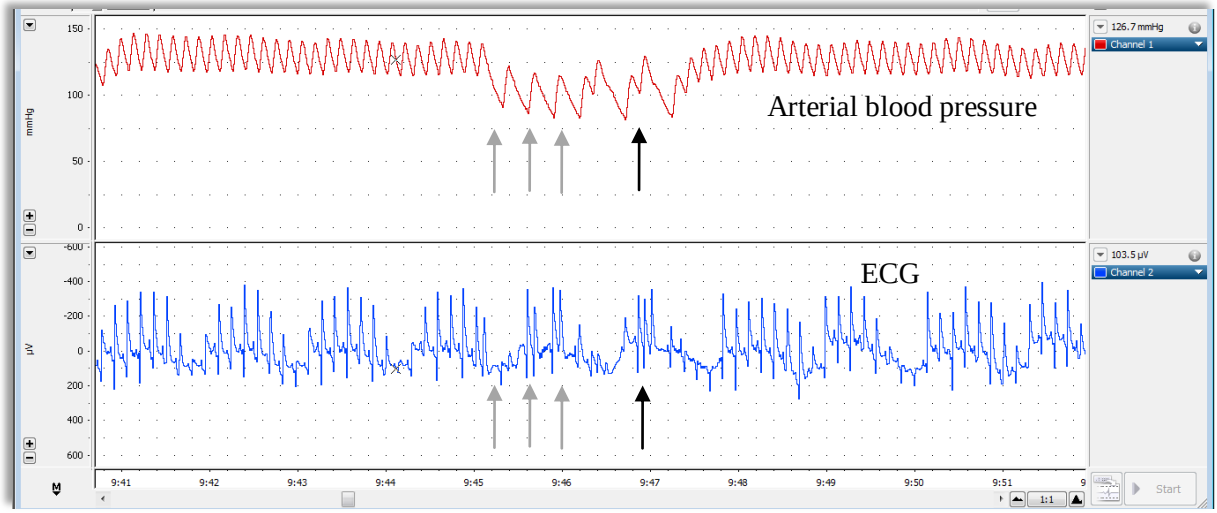

Figure 3.7 Salvos. A typical trace showing salvos (triplet), as indicated by the black arrows, with the associated fall in MABP. Note the single VPBs, as indicated by the grey arrows.

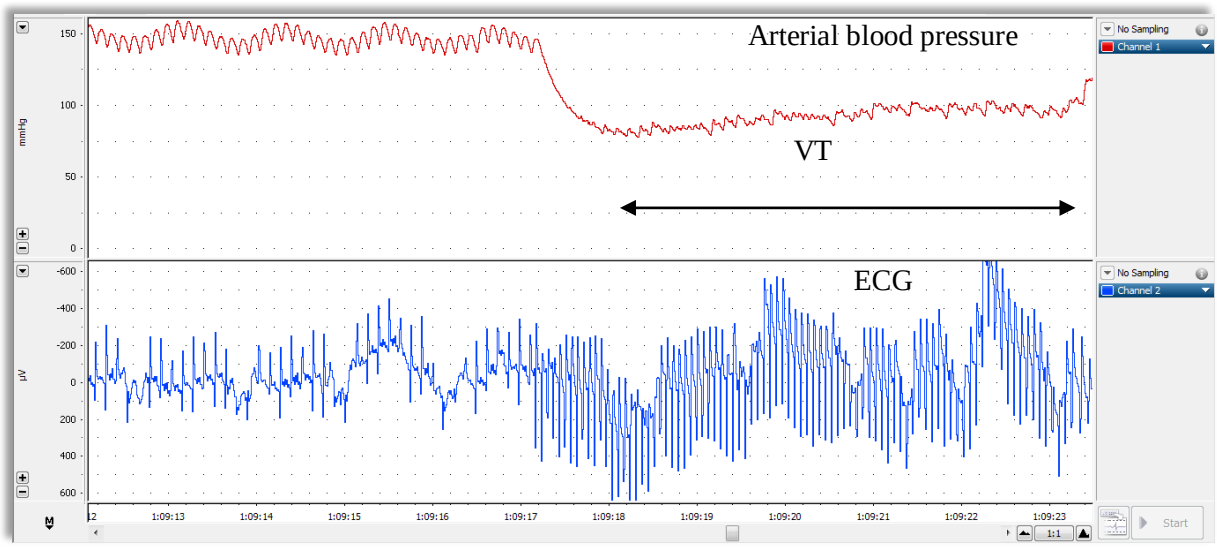

Figure 3.8 Ventricular tachycardia (VT). A typical trace showing VT, as indicated by the arrows, with the associated fall in MABP.

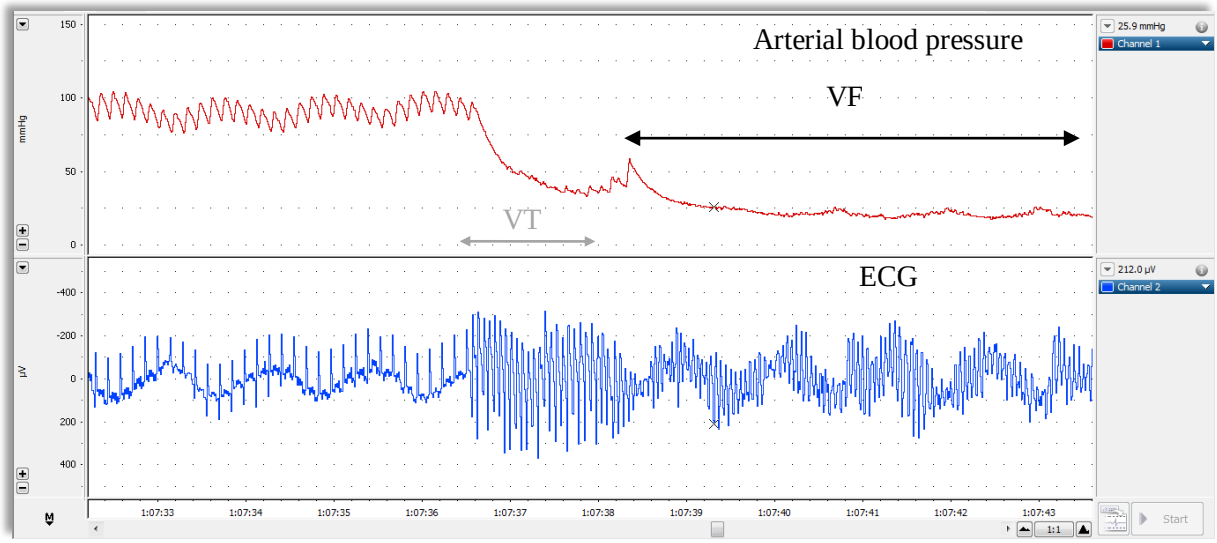

Figure 3.9 Ventricular fibrillation (VF). A typical trace showing VF, as indicated by the arrows, with the associated fall in MABP. VF is preceeded by a short period of VT, as indicated by the grey arrow.

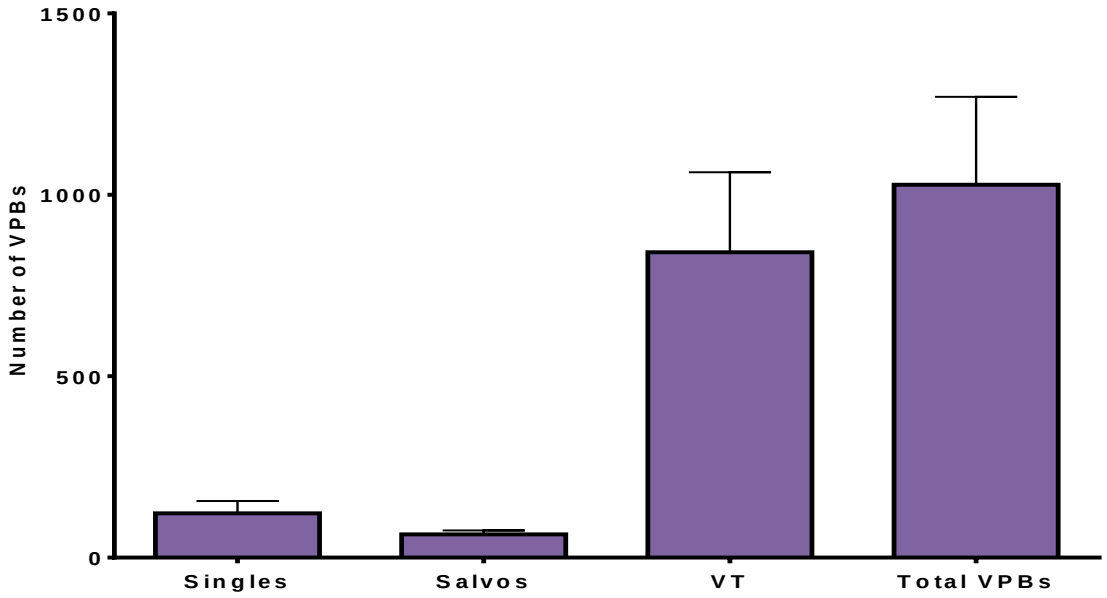

Figure 3.10 Quantification of ischaemia-induced ventricular arrhythmias. The total numbers and types of ventricular ectopic activity during 30 minutes of ischaemia, following ligation of the coronary artery. Data is expressed as mean ± SEM (n=8).

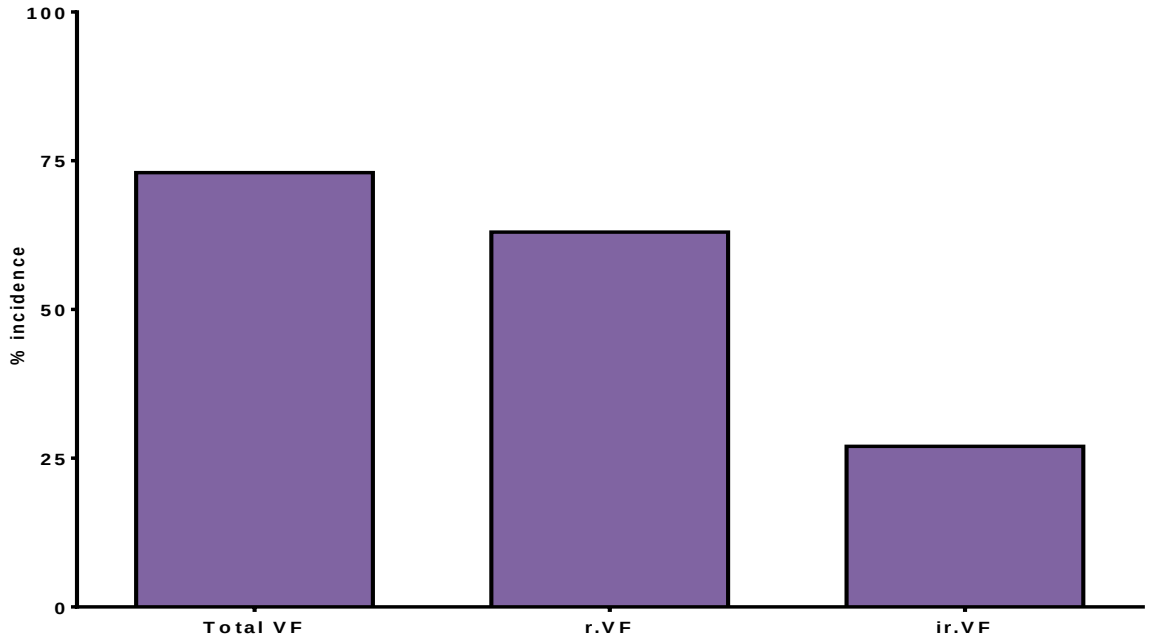

Figure 3.11 Incidence of reversible VF (r.VF) and irreversible VF (ir.VF). The % incidence of r.VF and mortality post-ligation of the LAD coronary artery. Data is expressed as % of n (n=11).

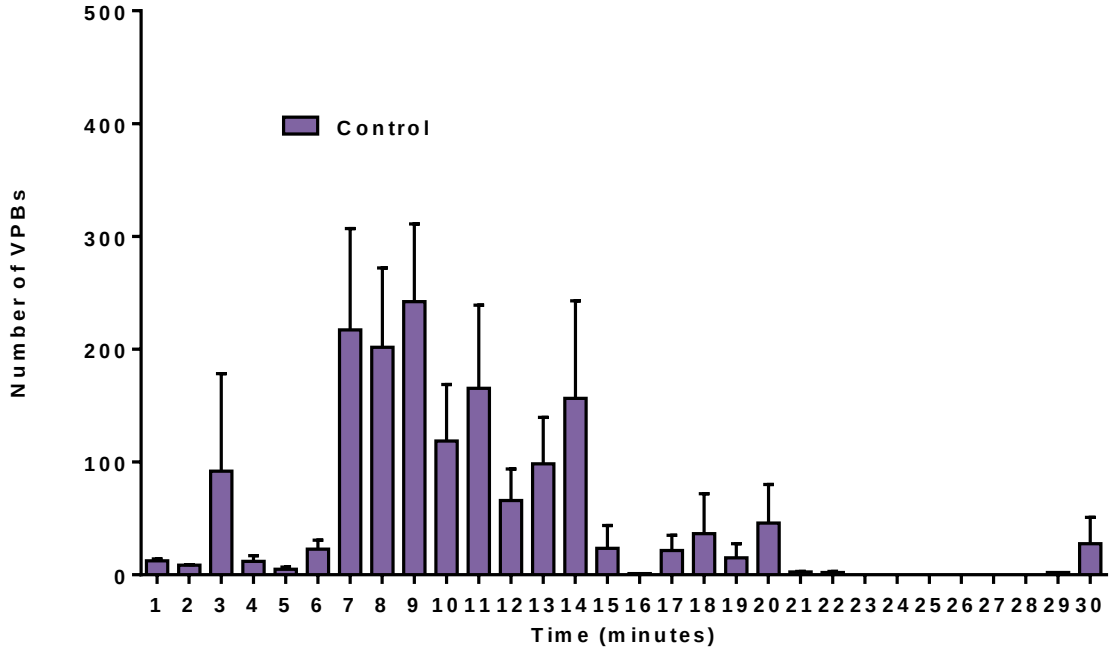

Figure 3.12 Time distribution of ischaemia-induced ventricular arrhythmias. The number of VPBs observed over 30 minutes of ischaemia. Data is expressed as mean ± SEM (n=8).

3.5 Discussion

3.5.1 Animal models of myocardial ischaemia

Animal models that mimic MI can help identify the mechanistic effect of CAO, and may assist in the development of novel cardioprotective agents. The predominant models used to investigate MI and reperfusion, are the *in vivo* coronary artery occlusion model and the Langendorff isolated perfused heart model primarily in small rodents, although many more are in existence (Abarbenell *et al*., 2010).

The *in vivo* coronary artery occlusion rat model, as employed in this study, has significant advantages over *in vitro* models in that it allows for the systemic responses to the trauma of ischaemia and reperfusion to be quantified. For example, use of an *in vivo* model allows the sampling of circulating blood for the analysis of platelet aggregability or the quantification of inflammatory cell infiltration (Verdouw *et al.*, 1998; Black, 2000; Vidavalur *et al*., 2008; Abarbanell *et al*., 2010), in addition to quantification of the occurrence of ischaemia and reperfusion-induced ventricular arrhythmias. In these present studies the rat was employed for two reasons; firstly because a previous study in our laboratory investigating the potential anti-arrhythmic effects of CBD was performed in rats (Walsh *et al*., 2010), thus allowing direct comparison with the intervention studies in this thesis; second, the rat represents a consistently reliable model of ischaemia-induced arrhythmias because of the inherent absence of extensive coronary collaterals, thereby ensuring severe ischaemia in the myocardium downstream of the ligation site (Johns and Olson, 1954). The *ex vivo* Langendorff isolated heart model, the antecedent of which was developed in 1866 (as reviewed by Skrzypiec-Spring *et al*., 2007; Zimmer, 1998), relies upon retrograde perfusion of the heart with physiological solution (e.g. Krebs Henseleit buffer) through an aortic cannula. Pressure transduction by way of the intraventricular balloon allows for quantification of pressure related-parameters; left ventricular developed pressure, end diastolic pressure, contraction rate and relaxation rate. In this model either global or regional ischaemia can be induced in a system devoid of systemic influences (Abarbenell *et al*., 2010). With respect to this study, the *in vivo* coronary artery occlusion model was utilised, because it was important to maintain influences of the systemic circulation and central and autonomic nervous systems because cannabinoids receptors are present at these sites in addition to on cardiomyocytes themselves and we were not sure where the cardioprotection afforded by CBD was a local or more systemic effect in the outset.

3.5.2 Study findings

As part of this study it was necessary to first characterise the response to coronary artery occlusion in the anaesthetised rat since the severity of arrhythmias can vary from operator to operator. Ligation of the left anterior descending coronary artery resulted in a significant fall in MABP (55%) when compared with the MABP of the stabilisation period. The same effect was not observed with respect to HR. With respect to MABP, these data are consistent with previously published studies e.g. Clark et al. (1980) who, during development of this model of coronary artery ligation, observed a significant fall in MABP, with negligible effects observed on pre- vs. post-ligation HR. This is further substantiated by other published data (Johnston *et al*., 1983), and these data are consistent with results from other studies performed in this laboratory (Walsh *et al.,* 2009; Walsh *et al.,* 2010). The mechanism of the fall in MABP upon ligation of a coronary artery is most likely due to a decrease in stroke volume due to a decreased force of contraction induced by the ion perturbations.

Arrhythmias occurring following ligation of the coronary artery were quantified by type and a vast proportion of the arrhythmias observed in the 30 minute occlusion period occurred in the form of VT. These data are in concordance with existing literature (Johnston *et al*., 1983; Crockett *et al*., 2000; Canyon and Dobson, 2004; Kloner et al., 2011). The mechanism of genesis of VT is postulated to be reentry. Within 15 to 30 seconds following induction of ischaemia, electrophysiological perturbations begin to manifest. Observable ECG changes at this time include depression of the TQ segment and elevation of the ST segment. The events hypothesised to engender these perturbations are of a metabolic and ionic origin, for example; extracellular accumulation of K^+, intracellular proton accumulation, intracellular Ca^{2+} overload, fatty acid accumulation and augmented circulating catecholamine concentrations (Corr and Sobel, 1979; Hirche *et al*., 1980; Janse and Kleber, 1981; Nayler, 1981; Kanayama *et al*., 1982; Sugiyama *et al*., 1982; Weiss and Shine, 1982; Kodama *et al*., 1984; Fozzard and Makielski, 1985; Reimer and Ideker, 1987).

Ligation of the coronary artery also induced VF, the total incidence of which was 73%. This is corroborated by the study by Canyon and Dobson (2004), which presented an incidence of 75% VF in the control group, although this group observed an incidence of irreversible VF of 58% whereas in this present study the incidence of irreversible VF was recorded as 27%. Mortality from VF is, however, variable across studies and has been reported to range between 5 and 46% (Crockett *et al*., 2000; Johnston *et al*., 1983; Philp *et al*., 2006). Mortality as a result of irreversible VF has previously been identified as being correlated with HR, in as much as HR was higher in those animals that experienced irreversible VF compared with those that did not (Bolli, Fisher and Entman, 1986). No such correlation was observed in our study (455 vs. 492 BPM; rats with irreversible VF vs. those without).

This correlation, in terms of HR and VF incidence, is clearer in dogs than in rats (Johnston et al., 1983; Bolli et al., 1986). The incidence of reversible VF (rat hearts can spontaneously revert to sinus rhythm) observed in this study was 63%, which is slightly greater than observed in comparable studies, where reversible VF incidence was found to be between 42% and 55% (Johnston *et al*., 1983; Crockett *et al*., 2000; Philp *et al*., 2006). Variability in the incidence of reversible VF may be due, in part, to operator dependent differences in placement of the ligature, since a ligature placed higher will increase the area at risk and concurrently the incidence of VF. In this study, death due to surgery, be it excessive post-surgery bleeding or premature coronary occlusion induced by trauma during ligature placement, was greater (31%) compared with a similar experimental procedure employed by Johns and Olson (1954), in which they observed an incidence of operative deaths of 21%.

Induction of experimental MI resulted in the onset of a relatively intense period of ventricular ectopic activity, which typically occurred around 5 minutes after cessation of regional coronary circulation, which is consistent with previous studies (Philp *et al*., 2006). A small burst of ectopic activity was also observed within the first minute of ligation; however, it is more likely that this arrhythmic activity is induced by the mechanical ligation process, as opposed to early onset of ischaemia-induced arrhythmias. Peak ectopic activity was observed between 7 and 20 minutes post-ligation. Indeed, these results are similar to that observed in comparative studies. In the anaesthetised rat model established by Clark et al. (1980), onset of ventricular ectopic activity was observed from 5 minutes after induction of ischaemia. Furthermore, peak arrhythmias in the study were observed to occur between 4 and 18 minutes after ligation. The slightly later onset of peak activity observed in the present study when compared with the Clark et al., (1980) study may purely be a result of operator dependent differences in technique. As with previous studies (Clark *et al*., 1980; Johnston *et al*., 1983), ventricular ectopic activity was evident in both phase Ia and phase Ib, the former having an electrophysiological mode of onset and the latter, brought on by a flux in circulating catecholamine concentrations (Kaplinsky *et al*., 1979; Janse and Kleber, 1981; Russell *et al*., 1984).

There are two distinct phases of ventricular arrhythmia following acute ischaemia and each phase, although temporally related, is distinct in aetiology. These phases, termed phases Ia and Ib are separated by a period free of VPBs (Harris, 1948). This period of quiescent ectopic activity was not observed in this study, with arrhythmias being recorded during both phases. This may be in part due to the distribution of arrhythmias into distinct phases being more evident in the dog, than rat (Kaplinsky *et al*., 1979; Russell *et al*., 1984; Janse *et al*., 1999).

In summary, these preliminary experiments have shown that in response to ligation of the left anterior descending coronary artery in anaesthetised rats, there is an immediate fall in MABP and the onset of ventricular ectopic activity within minutes post-occlusion, the severity/magnitude of both being

similar to those reported previously in the literature. Moreover, this same model has been shown to be sensitive to arrhythmia suppression by cannabinoid agents. Thus, it was confirmed that the model was a suitable method for subsequent experiments assessing the effect of cannabinoid drug interventions on ventricular arrhythmias.

4. Characterisation of the pharmacology of CBD *in vivo*

4.1 Introduction

4.1.1 The complex haemodynamic responses to cannabinoids

In addition to the well-documented psychotropic effects of cannabinoids, they can also cause complex changes in blood pressure (BP) which are dependent on; species, cannabinoid and type of anaesthesia (Malinowska, Baranowska-Kuczko and Schlicker, 2011). What is more, the endogenous cannabinoids, AEA and 2-AG, and the cannabinoid receptors (CB_2 and CB_2) have been identified as potential mediators of the pathogenesis of several cardiovascular conditions, namely, hypertension and atherosclerosis and myocardial infarction (Batkai and Pacher, 2009; Malinowska, Baranowska-Kuczko and Schlicker, 2011). Given the BP changes induced by cannabinoids and the potential role endocannabinoids play in cardiovascular disease, the ECS has become a source of interest with respect to treating pathologies of the cardiovascular system.

In anaesthetised rats, the endocannabinoid AEA, when administered by I.V. bolus injection, causes triphasic changes in cardiovascular parameters (Malinowska, Baranowska-Kuczko and Schlicker, 2011). Specifically, transitory bradycardia and associated hypotension with a successive pressor response and finally, a protracted depression of BP (Jarisch and Richter, 1939; Szolcsanyi *et al*., 1991; Varga *et al*., 1995; Lake *et al*., 1997; Malinowska, Kwolek and Gothert, 2001; Hogestatt and Zygmunt, 2002; Wheal *et al*., 2007).

The triphasic changes in cardiovascular parameters have been shown to be mediated by different receptors. Phase I is defined by a pronounced but transient bradycardia and fall in BP (also known as the Bezold Jarisch reflex; Lupinski *et al*., 2011), which is coupled with a fall in cardiac contractility and an increase in TPR. This phase was shown to be absent in $TRPV1^{-/-}$ mice (Pacher *et al*., 2004), can be blocked by a selective TRPV1 receptor antagonist and induced by a TRPV1 agonist (Malinowska *et al*., 2001; Pacher *et al*., 2004), suggesting that this phase was induced by activation of the vanilloid TRPV1 receptor (Malinowska, Baranowska-Kuczko and Schlicker, 2011).

Phase II of the triphasic changes induced by bolus AEA administration consists of a brief pressor response which is not a causal effect of the preceding hypotension of phase I (Varga *et al*., 1995; Kwolek *et al*., 2005), but more likely partially due to activation of TRPV1 receptors as it was again shown to be absent in $TRPV1^{-/-}$ mice. Additional receptors have also been implicated in the mediation of phase II, including the L-type Ca^{2+} channel (Kwolek *et al*., 2005; Malinowska, Baranowska-Kuczko and Schlicker, 2011).

It has been shown that the main mechanism responsible for the prolonged hypotension which characterises phase III is activation of the CB_1 receptor, as there was attenuation of the hypotension in $CB_1^{-/-}$ mice (Jarai *et al*., 1999; Ledent *et al*., 1999) and following the administration of either rimonabant or AM251 (Varga *et al*., 1995; Varga *et al*., 1996; Vidrio *et al*., 1996; Lake *et al*., 1997; Malinowska *et al*., 2001; Kwolek *et al*., 2005; Zakrzeska *et al*., 2010; Malinowska, Baranowska-Kuczko and Schlicker, 2012). It is thought that the CB_1 receptors that mediate the phase III hypotension in response to AEA are presynaptic CB_1 receptors located within the sympathetic nervous system (Varga *et al*., 1996; Szabo *et al*., 2001). However, it has also been suggested that postsynaptic CB_1 receptors located in the myocardium induce a negative ionotropic response which may also, in part, mediate phase III (Bonz et al., 2003; Pacher et al., 2004; Batkai and Pacher, 2009; Malinowska, Baranowska-Kuczko and Schlicker, 2012).

Marked differences exist in the haemodynamic responses to cannabinoids observed in rodents depending on whether they are conscious or anaesthetised. This is mostly due to the prevailing inhibitory effects of anaesthesia on numerous phases of the haemodynamic response (Malinowska, Baranowska-Kuczko and Schlicker, 2012). In conscious rodents, there is a brief pressor response to AEA, with additional bradycardia and hypotension at high doses (Lake *et al*., 1997; Gardiner *e al*., 2001; Gardiner *et al*., 2002; Gardiner *et al.,* 2009; O'Sullivan *et al*., 2007), while the protracted hypotension of phase III is absent in conscious rodents (Malinowska, Baranowska-Kuczko and Schlicker, 2012). There are also significant regional differences in the haemodynamic responses to AEA. In rat isolated mesenteric and hepatic arteries, AEA induced vasodilatation (Zygmunt *et al*., 1999), while in conscious rats, AEA produced transient vasoconstriction of renal, mesenteric and hindquarter vascular beds (Gardiner *et al*., 2002).

4.1.2 Non-vanilloid, non-CB_1 receptor-mediated haemodynamic responses

Some cannabinoid ligands have been shown to modulate blood vessel function through sites independent of CB_1 or vanilloid receptors (Jarai *et al*., 1999; Wagner *et al*., 1999; Ho and Hiley, 2003; Ho and Hiley, 2004; Johns *et al*., 2007; Unpublished findings Wainwright *et al*.).

Abn-CBD, a synthetic analogue of the CBD, induces hypotension *in vivo* and relaxation of isolated blood vessels from mice devoid of the CB_1 and CB_2 receptors implying an action at a vascular site other than the 'classical' cannabinoid receptors (Jarai *et al*., 1999; Ho and Hiley, 2004). Although this vasorelaxation is sensitive to SR141716A, it is not sensitive to other potent CB_1 selective antagonists such as HU-210, suggesting that this vascular site is potentially a third cannabinoid receptor (Jarai *et al*., 1999). The recently de-orphanised GPCR GPR55 was initially purported to be the novel

cannabinoid receptor responsible for the vasoactive effects of abn-CBD (Hiley and Kaup, 2007; Ryberg *et al*., 2007) since the GPR55 agonist, O-1602, causes vasorelaxation through an endothelium-dependent and SR-141716A sensitive mechanism (Jarai *et al*., 1999). Furthermore, although the GPR55 antagonist CBD does not induce hypotension itself (Adams *et al*., 1977), it does inhibit the vasorelaxation induced by abn-CBD (Jarai *et al*., 1999). However, Johns et al. (2007) cast doubt on the likelihood of GPR55 as the novel cannabinoid receptor having shown that I.V. administration of abn-CBD in $GPR55^{-/-}$ mice resulted in a rapid hypotension akin to that in WT, controls. Furthermore, vasorelaxant responses to both abn-CBD and O-1602 were of similar magnitude in mesenteric arteries taken from $GPR55^{-/-}$ and WT control mice. That being said, recent data from our laboratory has shown GPR55 receptor activation in WT mice can induce a vasodepressor response which is absent in $GPR55^{-/-}$ mice (unpublished findings Wainwright *et al*). Moreover, CBD induces vasodilator responses in isolated mesenteric arteries (Ho and Hiley, 2004), although it has no apparent hypotensive effect *in vivo* (Adams *et al*., 1977; Jarai *et al*., 1999)

4.2 Aim

Much of the contention around whether or not GPR55 mediates vasodepressor responses appears to arise from the nature of the ligands used, as it is now becoming apparent that some "selective" CB_1 ligands also possess activity at GPR55 receptors, and vice versa for GPR55 ligands (Ryberg *et al*., 2007; Thomas *et al*., 2007). In order to better understand which receptors are involved in mediating the previously reported anti-arrhythmic effects of CBD (Walsh *et al*., 2010), this initial study was undertaken to clarify the cardiovascular pharmacology of CBD *in* vivo. To do this I investigated whether CBD was acting at the CB_1 receptors by comparing it with a known CB_1 receptor agonist (ACEA) both in the presence and absence of a CB_1 receptor antagonist (AM251). Then to confirm whether, as the literature suggests, CBD acts as a GPR55 antagonist, I sought to block the effects of O-1602 (a proposed GPR55 agonist) with CBD.

4.3 Methods

4.3.1 Investigation of the effect of CBD and AM251 on CB_1 and GPR55 mediated haemodynamic responses

Following stabilisation, control BP and HR responses to bolus doses of ACEA (3mg kg^{-1}) or vehicle (tween 80, dimethyl sulfoxide (DMSO) and NaCl in the ratio 1:2:8, respectively) were obtained (n=8-14). Once the BP had returned to normal (~25 min), AM251 (1 mg kg^{-1}) was given and a response to ACEA obtained 10 minutes later. Following normalisation of BP, a third response to ACEA was then obtained following a higher dose (3 mg kg^{-1}) of AM251. In a separate group of rats (n=8) this protocol was repeated in the presence CBD (50µg kg^{-1}), which was given 10 min prior to the administration of the control dose of ACEA. As described in section 2.2 the cardiovascular variables of each animal were recorded throughout using LabChart 5 software.

4.3.2 Investigation of the effect of ACEA and O-1602 on CB_1 and GPR55 mediated haemodynamic responses

The protocol followed was as described above with the exception that O-1602 (30ng kg^{-1}; chosen from the literature) was employed in place of ACEA (n=4 for AM251 alone; n=6 for AM251 + CBD).

4.3.3 Determination of the dose-dependent effects of O-1602 on cardiovascular variables

In light of the finding that the dose of O-1602 employed in the experiments described above was insufficient to induce a marked fall in arterial blood pressure, a dose-response study of the effects of O-1602 (5, 15, 30, 50, 65 and 100ng kg^{-1}; n=4) was performed. Intravenous bolus injections were given in increasing doses every 5-10 minutes (or until cardiovascular parameters had returned to pre-injection levels) and the responses measured. Each animal operated as its own control.

4.3.4 Data expression

To take account for variable response times to administration of cannabinoids on MABP, measurements were taken from 100 seconds prior to and 300 seconds post-administration. Changes in MABP from baseline were calculated as % change and the time course of MABP changes were plotted to allow area above the curve (AAC) analysis for depressor responses. All results are presented as mean values ± SEM.

4.3.5 Statistical analysis

All % change in MABP data were subjected to analysis by two-way ANOVA followed by a Bonferroni post-hoc test and all comparisons were made to the control responses. All AAC analyses were subject to a Student's test and comparisons were made with vehicle control responses. n indicates the number of animals which underwent an experimental procedure.

4.4 Results

4.4.1 The effect of CB_1 receptor activation on MABP and HR *in vivo*

Bolus I.V. ACEA (3mg kg^{-1}) administration produced a significant depressor response which peaked 60 seconds after administration (7987 ± 1172 (ACEA) vs. 482 ± 108 AAC (vehicle); $P<0.005$; Figure 4.1 and Figure 4.3). This effect was restricted to an effect on MABP with no observable effect on HR (Figure 4.2). Baseline MABP in these groups was 151 ± 3 and 134 ± 5 mmHg, vehicle control and ACEA, respectively. Baseline HR was 455 ± 13 and 422 ± 22 BPM, vehicle control and ACEA, respectively.

4.4.2 The effect of AM251 on the ACEA-mediated depressor effects *in vivo*

The CB_1 receptor antagonist AM251 attenuated the depressor response mediated by ACEA (Figure 4.3) in a dose dependent manner (4798 ± 1667 (1mg kg^{-1} AM251) and 1641 ± 1223 (3mg kg^{-1} AM251; $P<0.05$) vs. 7987 ± 1172 AAC (Control response)).

4.4.3 Investigation of the effect of CBD on CB_1 receptor-mediated hypotension

Pre-treatment with CBD appeared to ameliorate the ACEA-mediated fall in MABP, but this was not significant. In contrast, CBD inhibited the AM251-mediated blockade of the ACEA response (Figure 4.4).

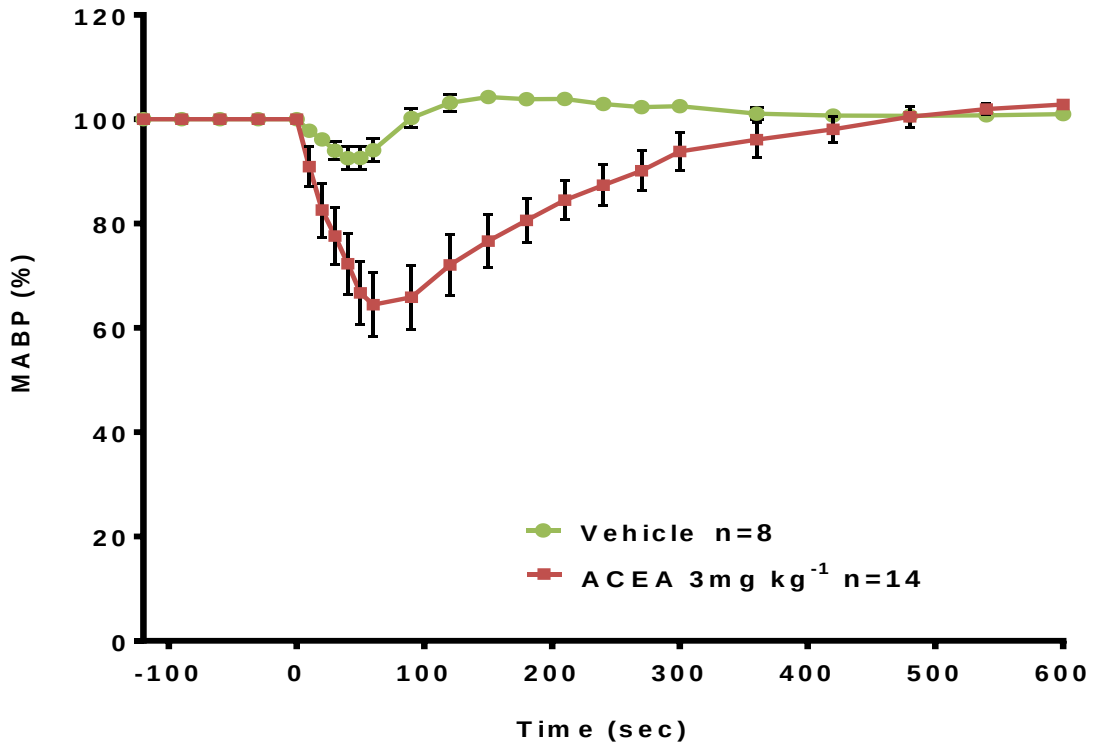

Figure 4.1 Effect of ACEA on MABP *in vivo*. Bolus ACEA (3mg kg^{-1}) I.V administration was made at 0 seconds. Data is expressed as mean % change in MABP ± SEM (n= 8-14).

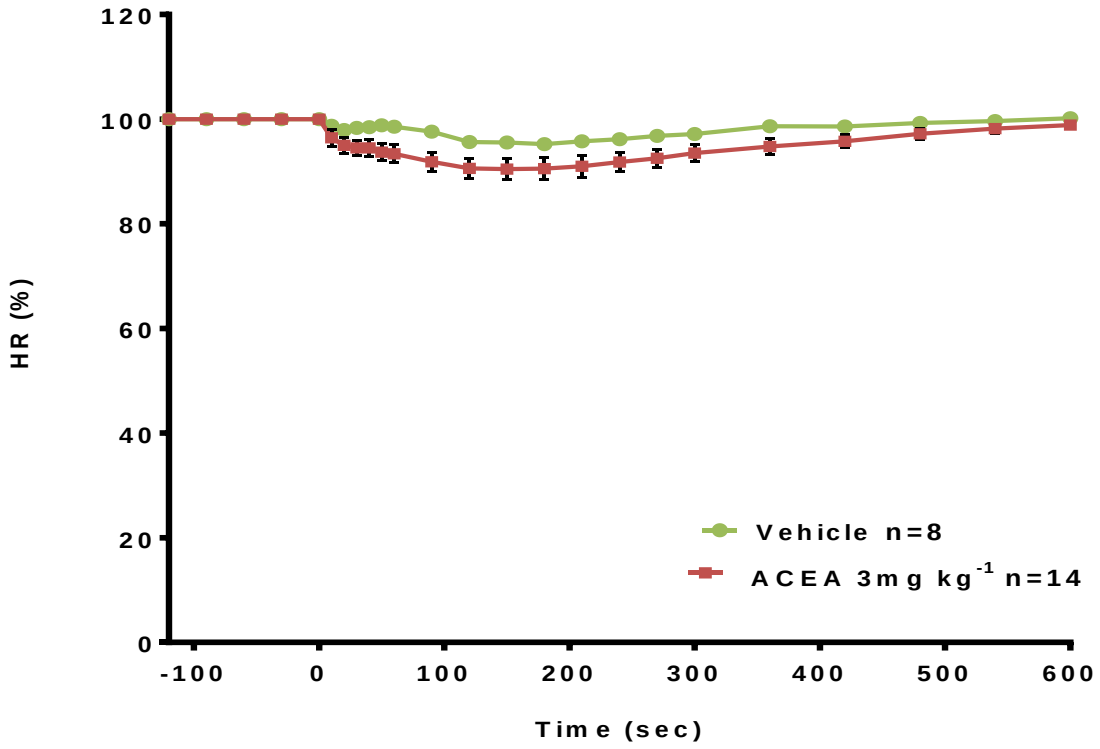

Figure 4.2 Effect of ACEA on HR *in vivo*. Data is expressed as mean % change in HR ± SEM (n=8-14).

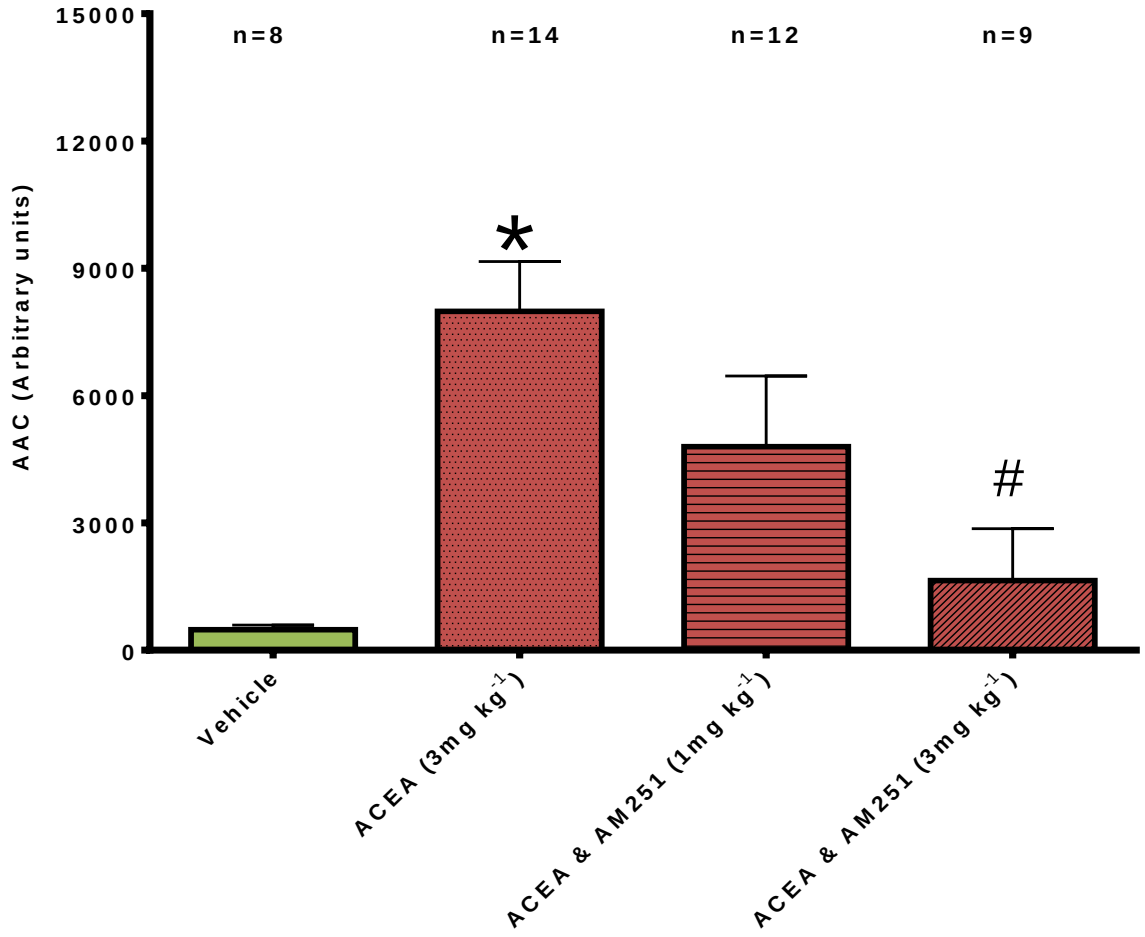

Figure 4.3 Effect of CB_1 receptor antagonism on area above curve (AAC) of MABP responses to ACEA. Data is expressed as mean AAC of MABP ± SEM (n=8-14). * indicates P<0.005 vs. vehicle and # indicates P<0.05 vs. ACEA (3mg kg^{-1}).

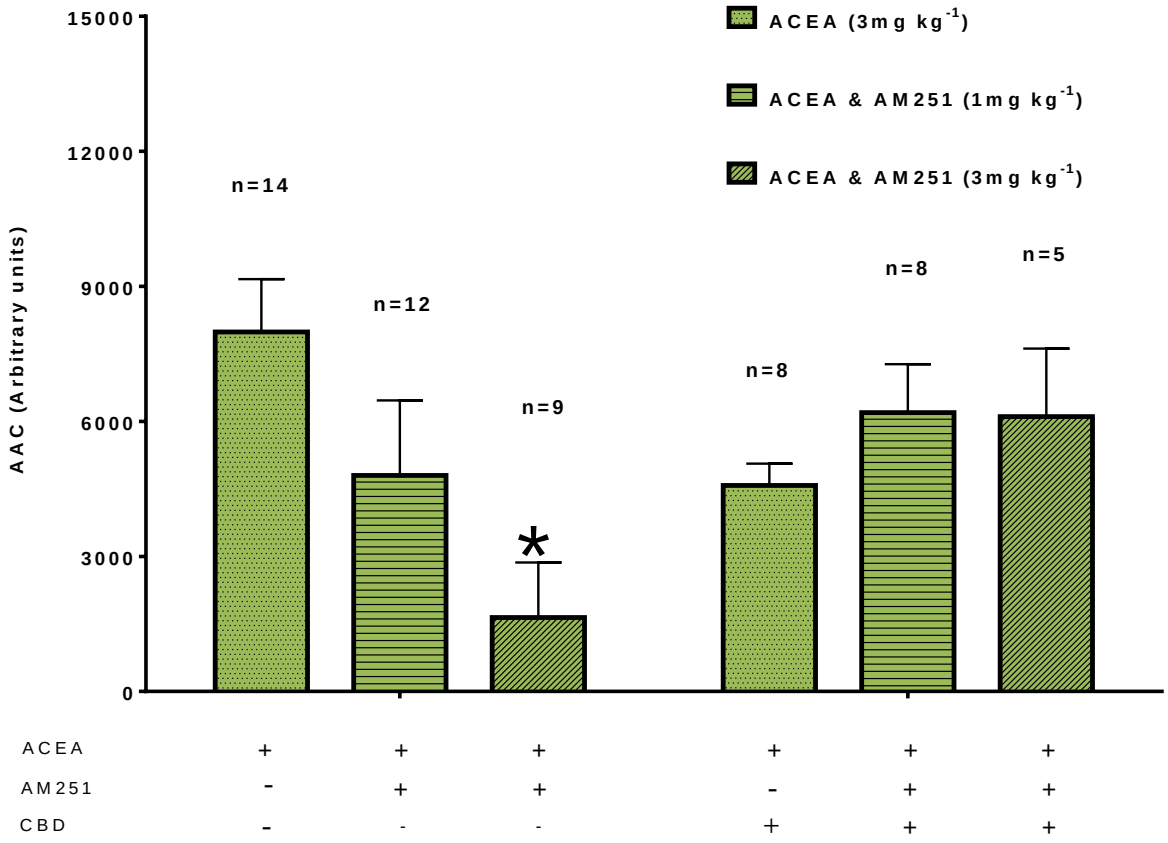

Figure 4.4 Effect of CBD and AM251 alone and in combination on the depressor responses to ACEA *in vivo*. Data is expressed as mean AAC ± SEM (n=5-14). * indicates P<0.05 vs. ACEA (3mg kg^{-1}) alone.

4.4.4 The effect of CBD on AM251-mediated hypotension

AM251 alone produced no significant change in MABP. However, the presence of CBD unmasked a pronounced dose-dependent depressor response to AM251, which was significant at the higher dose of AM251 ($P<0.001$; Figure 4.5).

4.4.5 Determination of the effect of AM251 and CBD on responses to O-1602

When tested across a wide dose range, O-1602 did not induce any changes in either MABP or HR in (Figure 4.6 and Figure 4.7). Although a single bolus dose of O-1602 (30ng kg^{-1}) produced a negligible haemodynamic response, a small depressor response was unmasked by AM251 at the highest dose ($p=0.09$; Figure 4.8). This depressor response to O-1602 was not observed when the rats were pre-treated with CBD (Figure 4.8).

4.4.6 Investigation of the effect of CBD on MABP and HR *in vivo*

CBD itself induced a small but significant depressor response upon bolus I.V. administration (1286 ± 324 vs. 316 ± 69; AAC; $P<0.05$; Figure 4.9, and Figure 4.11), whilst having no effect on HR (Figure 4.10). Baseline MABP in these groups was 151 ±3 and 146 ± 5 mmHg, vehicle control and CBD, respectively. Baseline HR in these groups was 455 ± 13 and 413 ± 9 BPM, vehicle control and CBD, respectively.

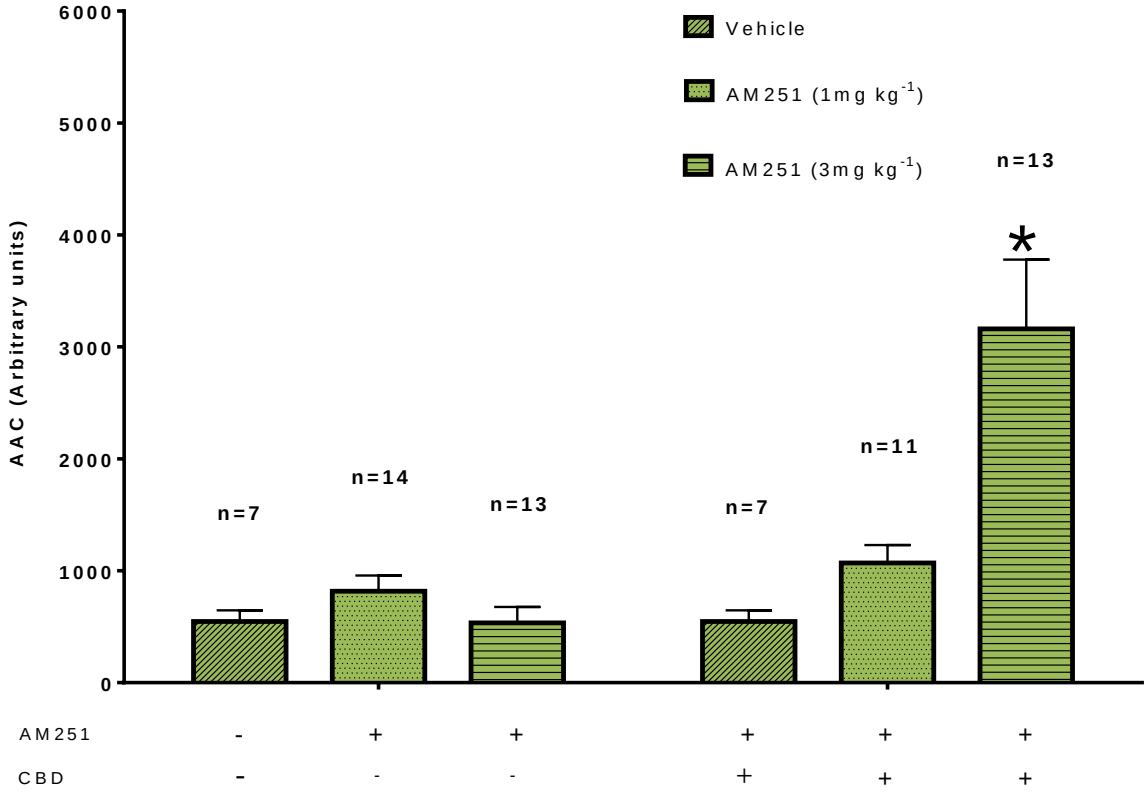

Figure 4.5 Effect of AM251 on MABP in the presence and absence of CBD. Data is expressed as mean AAC ± SEM (n=7-14). * indicates P<0.001 vs. vehicle and CBD.

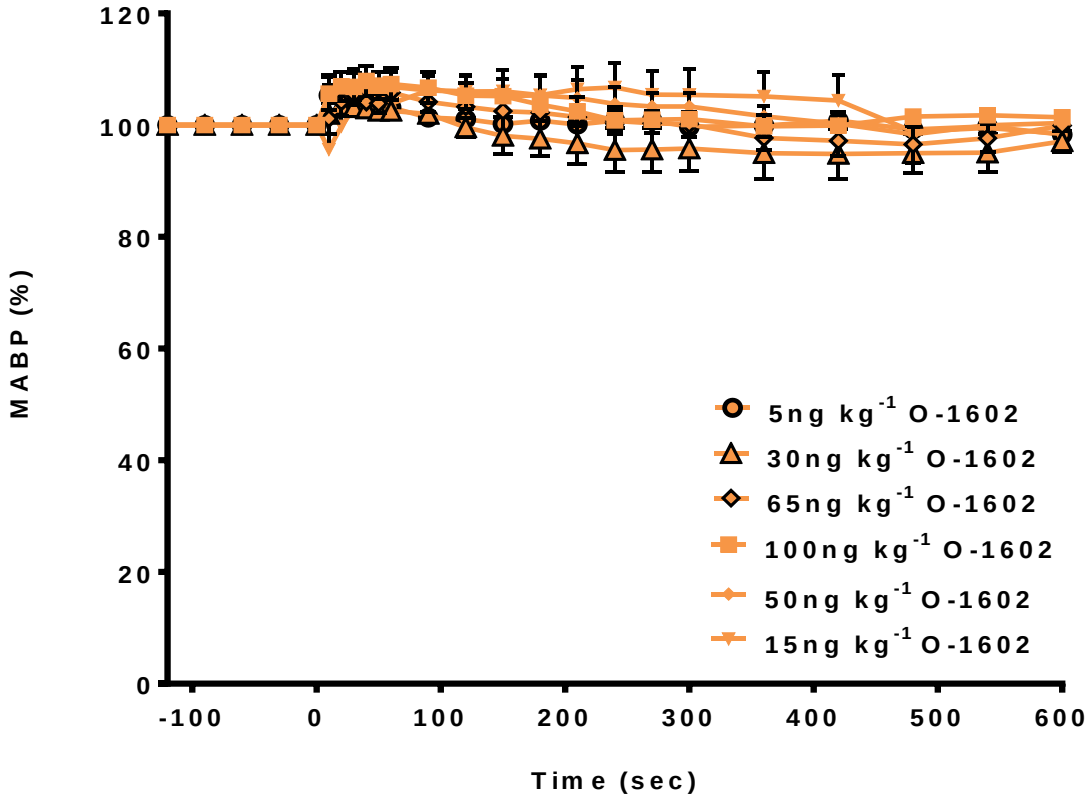

Figure 4.6 The dose-dependent effects of O-1602 on MABP *in vivo*. Data is expressed as mean % MABP ± SEM (n=4).

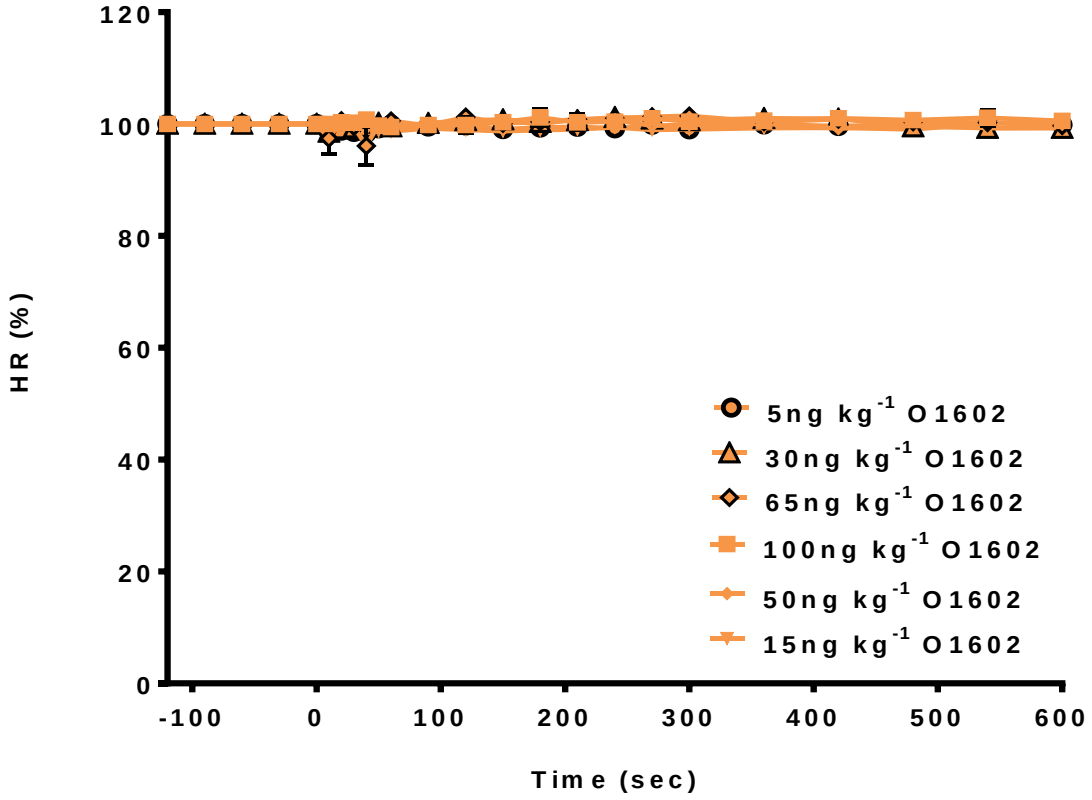

Figure 4.7 The dose-dependent effects of O-1602 on HR *in vivo*. Data is expressed as mean % HR ± SEM (n=4).

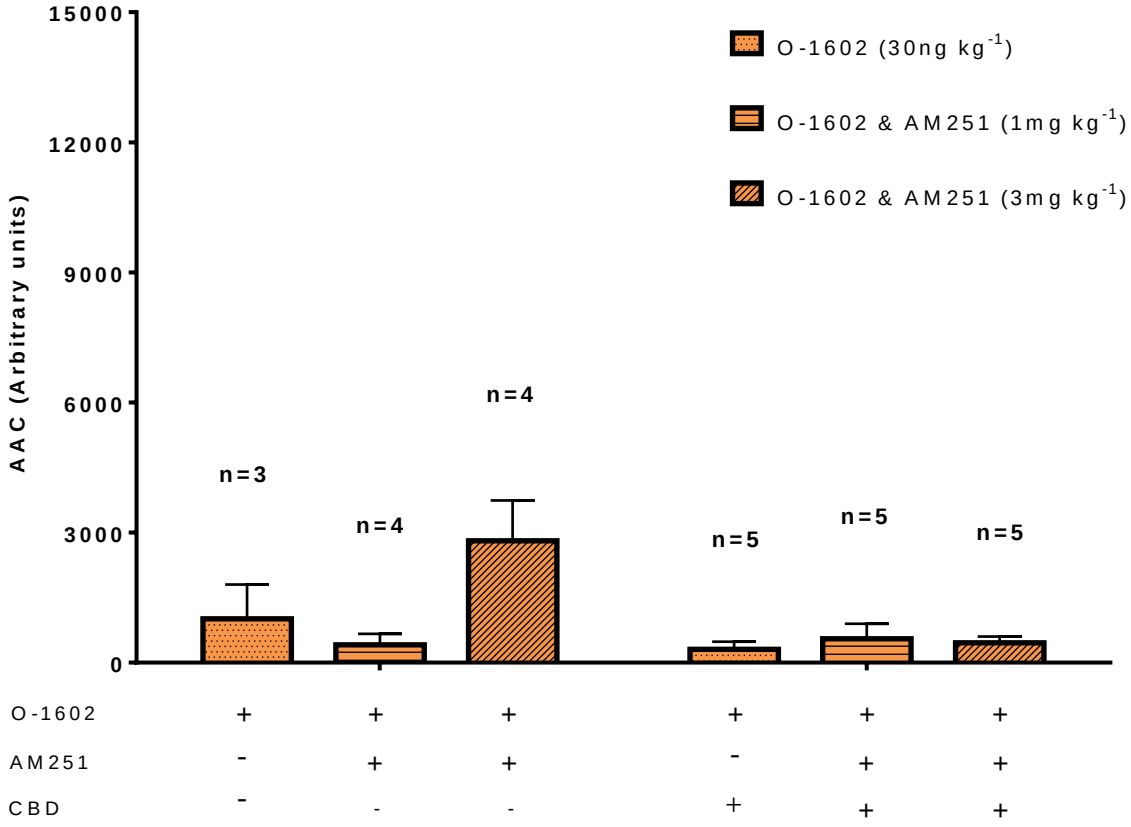

Figure 4.8 AAC of depressor responses induced by O-1602 in the presence of AM251 and CBD. Data is expressed as mean AAC ± SEM (n=3-5).

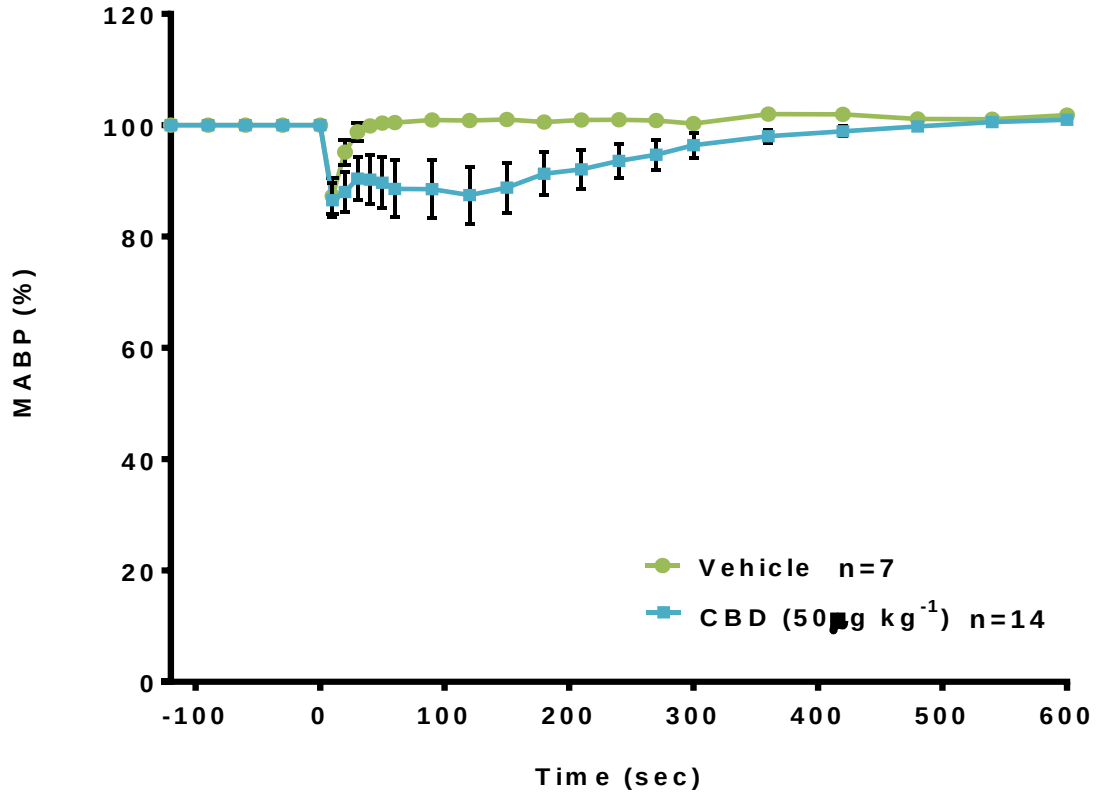

Figure 4.9 Effect of CBD on MABP *in vivo*. CBD (50µg kg^{-1}) was administered as a bolus dose (I.V.) at 0 seconds. Data is expressed as mean % change in MABP ± SEM (n=7-14).

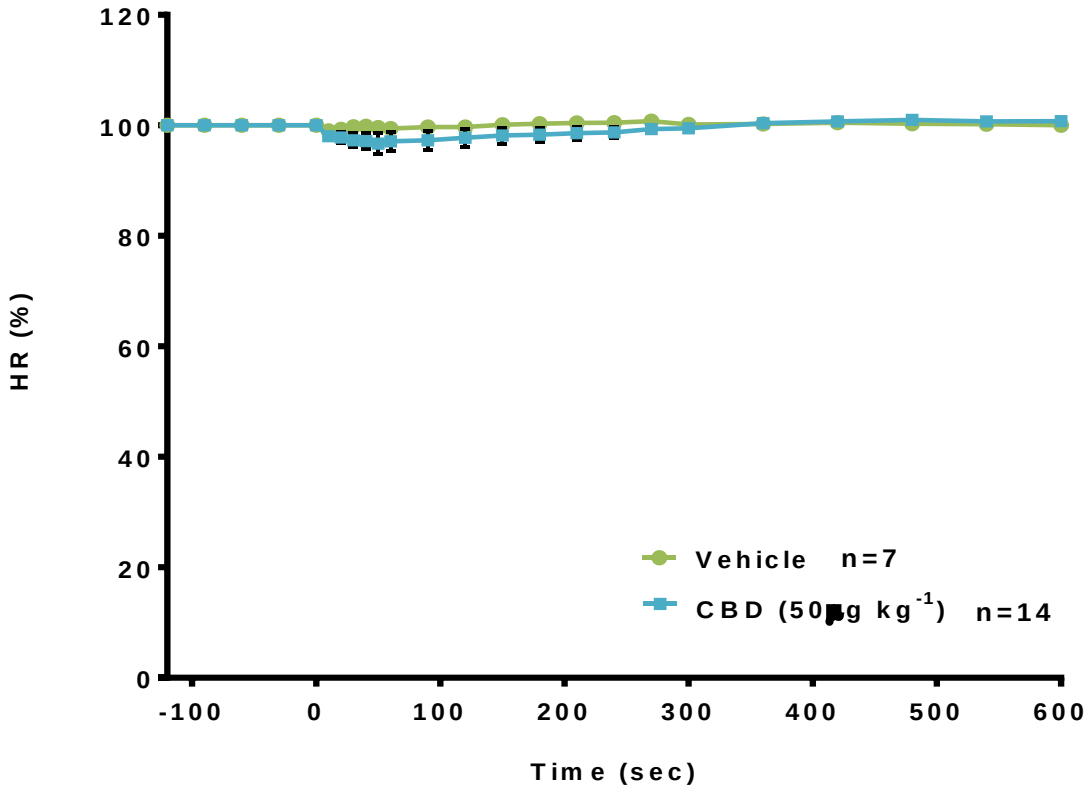

Figure 4.10 Effect of CBD on HR *in vivo*. CBD (50µg kg^{-1})was administered as a bolus dose at 0 seconds. Data is expressed as mean % change in HR ± SEM (n=7-14).

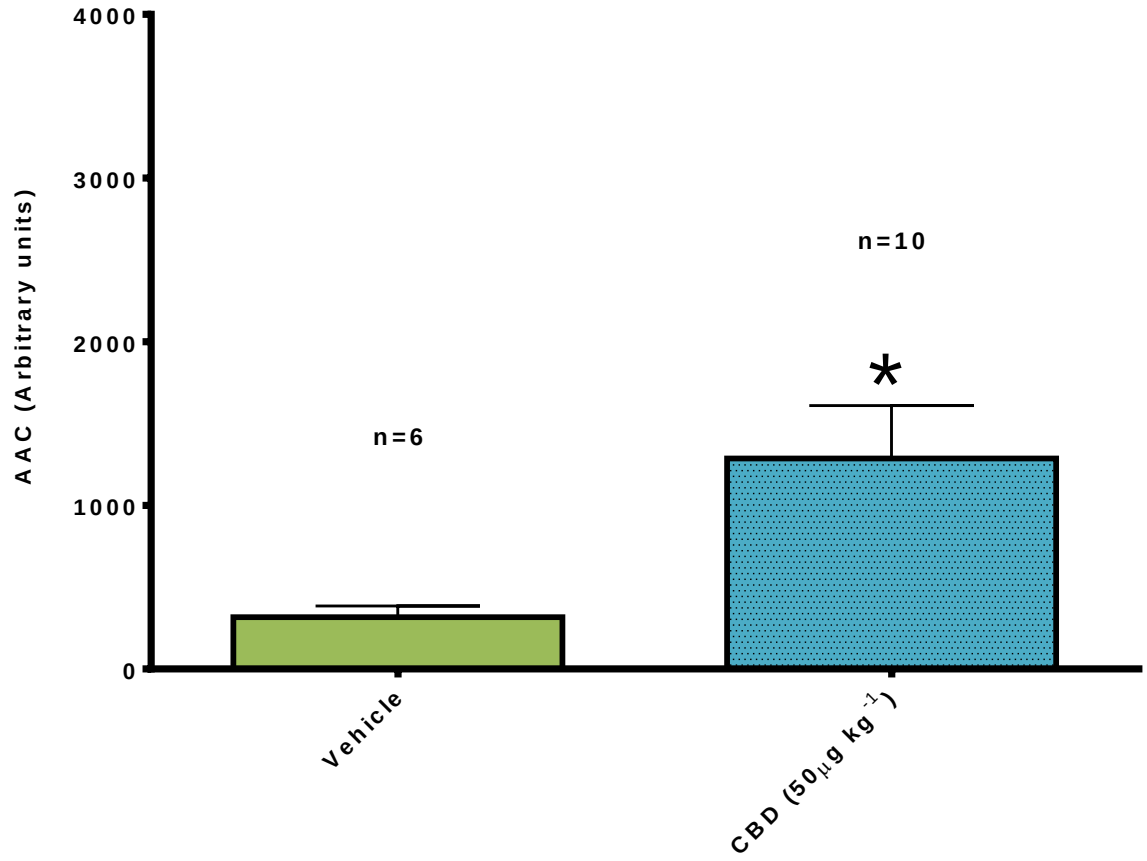

Figure 4.11 AAC of depressor responses to CBD *in vivo*. Data is expressed as mean AAC ± SEM (n=6-10). * indicates P<0.05 vs. vehicle.

4.5 Discussion

This series of experiments sought to better understand the involvement of CB_1 and GPR55 receptors in mediating changes in haemodynamic variables *in vivo*. In addition experiments were carried out to confirm that AM251 is a selective CB_1 receptor antagonist and furthermore, to establish the ability of CBD to block GPR55. These studies sought to inform the interpretation of the studies regarding the potential anti-arrhythmic effects of cannabinoids described later in this thesis. The data shows that in the anaesthetised rat the proposed GPR55 agonist, O-1602 did not significantly affect cardiovascular parameters. In contrast, ACEA-induced CB_1 receptor activation led to a significant depressor effect reversed by AM251. Administration of CBD not only abrogated the antagonistic effect of AM251 on ACEA-induced hypotension but also revealed an AM251-mediated depressor response which was absent when AM251 was administered alone.

4.5.1 Activation and antagonism of CB_1 receptors - the effect of MABP *in vivo*

The first finding from these experiments was that the CB_1 receptor agonist ACEA caused a fall in MABP in the anaesthetised rat while having no effect on HR, which may suggest that the former is due to a fall in peripheral vascular resistance (Haddy, Overbeck and Daugherty, 1968; Levick, 2003). Ford et al. (2002) showed that in an isolated rat heart model, bolus ACEA administration produced a fall in left ventricular developed pressure and coronary vasodilatation. Furthermore, Montecucco and Di Marzo (2012) showed that CB_1 receptor activation induces transient bradycardia and hypotension. It has been demonstrated previously that the coronary vasodilator haemodynamic responses to ACEA are unaffected by a CB_2 receptor agonist (JWH015) (Ford *et al.*, 2002), however, the effect of a CB_1 receptor antagonist on ACEA-induced hypotension *in vivo* has not been investigated until now. The depressor response induced by ACEA was blocked by the CB_1 receptor antagonist AM251 in a dose-dependent manner, providing evidence that the depressor effects of ACEA are mediated by the CB_1 receptor and that AM251 is acting as a CB_1 antagonist.

Although classified as a GPR55 antagonist (Ryberg *et al.*, 2007), CBD attenuated the hypotension induced by ACEA, although this was not statistically significant, suggesting that under the present experimental conditions it is exhibiting some activity as a CB_1 receptor antagonist. Indeed, it has been shown that CBD can act as a weak antagonist and inverse agonist at the classical cannabinoid receptors, CB_1 and CB_2 in mouse brain membranes and in CHO cells stably expressing CB_1 or CB_2 receptors (Thomas *et al.*, 2007). CBD was also found to reverse the ability of AM251 to block ACEA-

induced hypotension. Since both CBD and AM251 are pharmacologically active at CB_1, it is conceivable that pre-treatment with CBD prevents the inhibition of ACEA-induced vasodepression by AM251 because the receptor site is already occupied by CBD.

Interestingly, CBD itself induced a depressor response, which supports existing literature in which CBD was found to reduce mean, systolic and diastolic blood pressure in pithed rats (Zakrzeska *et al.*, 2010) and to induce vasorelaxation in rat aorta (O'Sullivan *et al.*, 2009). This is consistent with data from our own laboratory which has shown that CBD induces a depressor response in both WT and $GPR55^{-/-}$ anaesthetised mice (unpublished findings, Wainwright *et al.*), demonstrating that this effect is independent of an action at GPR55. Zakrzeska et al. (2010) also investigated the involvement of CB_1 receptors and the haemodynamic responses to AEA; they found that CBD antagonised the phase III effects of AEA, leading to the conclusion that both AEA and CBD were acting at a non-CB_1 vascular cannabinoid receptor.

4.5.2 Determination of the involvement of GPR55 in the mediation of haemodynamic responses

The consequences of GPR55 receptor activation with regard to blood pressure have not been fully characterised, although Johns et al. (2007) have shown that baseline BP and HR are not significantly different between WT and $GPR55^{-/-}$ mice, suggesting that GPR55 activation does not contribute to normal BP control. I have shown that the selective GPR55 agonist, O-1602 produced no discernible change in either MABP or HR in the anaesthetised rats across a wide range of doses, suggesting a lack of GPR55 receptors in the vasculature and/or myocardium. This is in contrast to the study by Johns et al. (2007) who found that O-1602 could reverse phenylephrine-induced tone in mouse mesenteric arteries. However, controversy exists regarding the ligand pharmacology of GPR55. For example, O-1602 has been shown to increase GPR55-dependent GTPγS signalling (Johns *et al.*, 2007) but not β-Arrestin-2 GPR55 complex formation (Kapur *et al.*, 2009). What is more, it is proposed that the primary pharmacological target of O-1602 is GPR18 and not GPR55 (McHugh and Ross, 2009). Although it was initially proposed that the vasoactive effects of O-1602 were mediated by GPR55, subsequent investigation of the pharmacology of GPR55 has led to speculation of the specificity of the ligands for GPR55 (Fischbach et al., 2007; Hiley and Kaup, 2007; Johns *et al.*, 2007; Ryberg *et al.*, 2007; Waldeck-Weiermair *et al.*, 2007; Kapur *et al.*, 2009; Sylantyev *et al.*, 2013). Therefore, it is possible that rather than reaching the conclusion from the present experiments that GPR55 activation does not induce hypotension, it might be a case that O-1602 is not causing activation of GPR55.

In the experiments to determine the effects of AM251 on ACEA-induced depressor responses it was identified that AM251 caused a transient fall in MABP. Although AM251 was initially used as a selective CB_1 receptor antagonist, since CB_1 receptor activation causes a transient bradycardia and prolonged hypotension (Montecucco and Di Marzo, 2012), the brief depressor response observed in response to AM251 cannot be rationalised as a CB_1-dependent effect, since CB_1 receptor antagonism would be expected to increase rather than decrease BP. Recent accounts of the pharmacology of this ligand suggest that it is not as selective as anticipated, as it is reported to possess potent agonist activity at GPR55 (EC_{50} = 39nM; Ryberg *et al*., 2007). Moreover, recent data from this laboratory has shown that, similar to the current observation in rats, AM251 induces a depressor response in WT mice. The absence of a response in $GPR55^{-/-}$ mice suggests that this effect is through an action GPR55 (unpublished observations, Wainwright *et al*.).

To confirm whether the transient hypotension caused by AM251 was GPR55 mediated, CBD was used as a GPR55 receptor antagonist. Interestingly, when CBD was given prior to AM251, there was a marked potentiation of the depressor response to AM251, which based on their reported opposing pharmacologies at GPR55 was unexpected and this may suggest that both AM251 and CBD have 'off target' effects beyond those their initial pharmacological profiles suggested. However, CB_1 receptors and GPR55 receptors are acknowledged to be co-localised (Kargl *et al*., 2012) and evidence exists in the literature which describes cross-talk between GPR55 and CB_1 receptors, through integrin clustering. In particular, when the integrins are unclustered, CB_1 receptor activation results in a signalling cascade which includes, but is not limited to, spleen tyrosine kinase (Syk), which in turn inhibits a key protein in the GPR55 signal transduction cascade. However, when integrins are clustered, Syk is no longer able to inhibit GPR55 signalling (Waldeck-Weiermair *et al*., 2008). As such, it is feasible that instead of acting as an antagonist at GPR55, CBD is acting at CB_1 receptors in a manner which relieves CB_1-receptor mediated inhibition of GPR55, thereby unmasking a GPR55-mediated vasodilator response to AM251.

4.5.3 Summary and conclusions

In summary, this series of experiments have shown that CB_1 receptor activation by ACEA results in a significant fall in MABP which can be dose-dependently inhibited by the CB_1 receptor antagonist and GPR55 receptor agonist, AM251. Furthermore, the data shows that both CBD and AM251 themselves can induce transient depressor responses by mechanisms that may be CB_1 mediated and GPR55-mediated, respectively. While the 'selective' GPR55 agonist, O-1602 does not induce hypotension in the anaesthetised rat, the lack of response may be due to the poorly defined pharmacology of this compound rather than an indication that GPR55 does not mediate a vasodilator response. Finally,

CBD has been shown to be capable of potentiating AM251-induced hypotension, suggesting possible cross-talk between these two receptors. Moreover, these findings suggest that neither AM251 nor CBD are selective antagonists for CB_1 or GPR55, respectively.

Taken together these findings led to the design of the subsequent series of experiments outlined in the next chapter of this thesis focusing on assessing the ability of each agent, alone and in combination, against ischaemia-induced arrhythmias rather than using AM251 simply as a pharmacological tool to rule out or confirm the role of CB_1 receptors in the cardioprotective effect of CBD.

5. Assessment of the effects of CBD and AM251 on ischaemic arrhythmias

5.1 Introduction

5.1.1 Cannabinoids and arrhythmias

Cannabinoid receptors are widely expressed in a multitude of tissues, including cardiomyocytes, where they can inhibit activity of adenylate cyclase (AC) and synthesis of cAMP, thereby cannabinoids may potentially play a significant role in arrhythmia initiation and propagation (Li and Ng, 1984; Lubbe *et al*., 1992; Pacher and Hasko, 2006). The endocannabinoid, AEA has been shown to protect against adrenaline-induced arrhythmias in a rat model, an effect unchanged by addition of CB_1 (SR141716A) or CB_2 (SR144528) receptor antagonist (Ugdyzhekova *et al*., 2001). Moreover, it has been identified that cannabinoids are involved in mediating cardioprotection. In addition, AEA and its metabolically stable analogue, R-(+)-methanandamide, reduce ischaemia and reperfusion-induced arrhythmias (Krylatov *et al*., 2002). More recently, Li *et al*. (2009 and 2012), showed that AEA mediates its anti-arrhythmic effects by suppressing AP duration, modulating LTCC function, suppressing I_{to} currents by a non-$CB_{1/2}$-dependent pathway and finally, by increasing I_{KATP} current by a CB_2-dependent pathway.

5.1.2 Cannabidiol and the cardiovascular system

CBD, the non-psychotropic derivative of *Cannabis sativa*, most abundant only after Δ^9-THC, was first isolated by Mechoulam and Shvo (1963). This phytocannabinoid has been shown to act in an anomalous manner at the CB_1 and CB_2 receptors, following observations that CBD was shown to produce dextral shifts in the [^{35}S] GTPγS binding dose-response curves of CP55940 and WIN55212, initially indicating non-competitive antagonist-like pharmacology. It was also observed that alone CBD had the capacity to inhibit [^{35}S] GTPγS binding, indicating inverse agonism at CB_1 and CB_2 (Thomas *et al*., 2007). In addition, CBD is an inhibitor of fatty FAAH (Wantanabe *et al*., 1996), an agonist at the 5-HT_{1A} receptor (Russo *et al*., 2005), and an antagonist of GPR55 (Ryberg *et al*., 2007). Although the exact pharmacology of CBD is still, as yet, not completely clear, existing evidence suggests that it may be tissue protective. Indeed, CBD is cerebroprotective following episodes of cerebral ischaemia, an effect attributed to reductions in neutrophil accumulation and inhibition of myeloperoxidase activity (Hayakawa *et al*., 2007). Furthermore, Durst et al. (2007) observed that in an *in vivo* model of MI and reperfusion, chronic CBD administration reduced infarct size. This effect was attributed to a reduction in inflammatory infiltration into the ischaemic and necrotic zones of the myocardium. Interestingly, this protective effect was not observed in a comparative *in vitro* model, further substantiating the role of systemic influences on the mediation of the observed protection. In

the acute phase of *in vivo* MI CBD is protective inasmuch as it can reduce ischaemia-induced ventricular arrhythmias and reduce infarct size (Walsh *et al*., 2010).

5.1.3 The putative cannabinoid receptor, GPR55

First isolated in 1999, the GPCR receptor, GPR55 has been identified as a putative cannabinoid receptor (Sawzdargo *et al*., 1999). The GPR55 receptor demonstrates only 13.5% sequence homology with the CB_1 receptor (Ryberg *et al*., 2007). Investigation of the functional fingerprints of both the classical cannabinoid receptors and GPR55 revealed that human GPR55 does not share a functional fingerprint with CB_1 and CB_2 receptors (Petitet, Donlan and Michel, 2006). Functional fingerprints are the key residues involved with dealing with the binding and/or activity of ligands. The ligands examined in the identification of the functional fingerprints of GPR55, CB_1 and CB_2 receptors were those purported to be active at all three receptors and as such the data would suggest that although GPR55 may be activated by cannabinoids, it does not possess a binding pocket similar to that of CB_1 and CB_2 receptors (Petitet, Donlan and Michel, 2006). Confirmed agonists of GPR55 are lysophosphatidylinositol (LPI), rimonabant and AM251 (Kapur *et al*., 2009). CBD can antagonise [^{35}S] GTPγS binding with AEA, CP55940 and O-1602 in HEK293 cells transfected with GPR55 (Ryberg *et al*., 2007). When activated GPR55 is coupled with either G_{13}, G_q and G_{12} and there is a resultant increase in cytosolic $[Ca^{2+}]_i$ in a IP_3-mediated manner (Ryberg *et al*., 2007; Lauckner *et al*., 2008; Henstridge *et al*., 2009; Kapur *et al*., 2009; Henstridge *et al*., 2010; Pertwee *et al*., 2010).

It has recently been demonstrated that activation of GPR55 by the proposed endogenous ligand, LPI, can, in cultured ventricular myocytes, induce Ca^{2+} mobilisation via LTCCs, IP_3 receptors or endo-lysosomes, depending on the site of activation and may therefore suggest a role for GPR55 in the myocardium. Moreover, the LPI-induced Ca^{2+} release from endo-lysosomes requires signalling via NAADP and can consequently increase SR Ca^{2+} load and this may infer that the recently de-orphanised receptor could modulate contraction of cardiomyocytes (Yu *et al*., 2013). Data from our laboratory has demonstrated that GPR55 expression or function is modified by ischaemia because LPI was only able to increase infarct size when global ischaemia in Langendorff perfused hearts, and not post-ischaemia (Robertson-Gray, unpublished). The complexity of the data so far gleaned from investigation of the pharmacology of GPR55 means that identification of a functional effect of GPR55 receptor activation in the cardiovascular system more intriguing.

5.2 Aim

Numerous studies have investigated the effects of cannabinoids in the cardiovascular system, including identification of the negative ionotropic effects of CB_1 receptor activation (Ford *et al*., 2002; Bonz *et al*., 2003) and examination of the variable vasodilator effects of the endocannabinoid AEA (Mukhopadhyay *et al*., 2002; O'Sullivan *et al*., 2004). Moreover, AEA has been demonstrated to reduce arrhythmia incidence, although it is not likely that CB_1 or CB_2 are involved (Ugdyzhekova *et al*., 2001; Krylatov *et al*., 2002; Li *et al*., 2009; Li *et al*., 2012). Phytocannabinoids, like CBD, have also been shown to be anti-arrhythmic, although the pharmacological target is not known (Walsh *et al*., 2010). CBD has a complex pharmacology including, but not limited to, a capacity to act as an antagonist at the recently deorphanised GPR55, a receptor whose role in the CVS is still not clear but may involve modulation of Ca^{2+}_i handling (Ryberg *et al*., 2007; Yu *et al*., 2013).

In the previous chapter we identified that neither CBD nor AM251 are selective antagonists for CB_1 and GPR55, respectively. Moreover, we demonstrated that AM251 appears to induce haemodynamic changes via GPR55 activation. Therefore, the aim of this series of experiments was to identify the ability of CBD and AM251, alone and in combination against ischaemia-induced ventricular arrhythmias.

	CBD	AM251
CB_1 receptor	Inverse agonist (Pertwee, 2003; Thomas *et al*., 2007) Antagonist (Pertwee, 2005)	Antagonist (Lan *et al*., 1999)
GPR55	Antagonist (Sylantyav *et al*., 2013)	Agonist (Ryberg *et al*., 2007; Henstridge *et al*., 2010)

Table 5.1 Pharmacological targets for CBD and AM251.

5.3 Methods

5.3.1 Surgical procedure for experimental coronary artery occlusion

Male Sprague Dawley rats (250-450g; n=30 before any exclusion criteria were applied) were prepared for surgery and experimental coronary artery occlusion as described in sections 2.2 and 2.2.1.

5.3.2 Assessment of haemodynamic parameters and occurrence of VPBs

Haemodynamic parameters and occurrence of VPBs were monitored throughout each experimental protocol, as described in 2.2.2 and 2.2.3. Quantification as described by the Lambeth conventions (Walker *et al*., 2013) was completed as described in 3.4.2.

5.3.3 Experimental Protocol

Figures 5.1-5.4 illustrate the experimental protocols used in this study. All animals were allowed a 25 minute stabilisation period before bolus drug administration at 5 minute intervals. Experimental groups were; (i) vehicle control (n=6), (ii) CBD (50µg kg^{-1}) alone (n=6), (iii) AM251 (1mg kg^{-1}) alone (n=6), (iv) CBD followed by AM251 (n=6), and (v) AM251 followed by CBD (n=6). The vehicle control was a combination of the vehicles used for CBD and AM251, which was EtOH and a tween, DMSO, NaCl mixture (in the ratio 1:2:8), respectively. After the final administration of the vehicle control or drug, induction of coronary artery occlusion was commenced by compressing the snare against the surface of the myocardium, thereby ligating the LAD coronary artery. A timetable of drug treatments was prepared in advance of completion of this series of experiments in order to randomise the completion of each drug intervention over the duration of this series of experiments. Moreover, the files for each experiment were labelled by date rather than drug treatment in order to maximise randomization of drug interventions.

5.3.4 Immunohistochemical staining for GPR55

In order to identify whether GPR55 receptors were present in the rodent myocardium, colleagues at AstraZeneca in Molndal, Sweden performed IHC staining for its detection, as described in section 2.3.2. Whole heart sections were fixed, processed and embedded before being cut at 3-4µm and

mounted on slides for IHC staining with a specific antibody directed against GPR55. The primary antibody, GRP55-A162T750 was diluted 1:750 using DaVinci Green Diluent before incubation with the myocardial sections. Staining was performed in sham (those which has undergone the full experimental procedure with the exception of having the heart exteriorized and ligature loosely placed around the LAD coronary artery) hearts, hearts subject to ischaemia by ligation of the LAD coronary artery and hearts subject to ischaemia but pretreated with CBD (50µg kg^{-1}).

5.3.5 Exclusion criteria

Any animal that displayed a starting MABP of less than 70mmHg, or those animals in which spontaneous arrhythmias developed in the stabilisation period, were discounted from further study. Furthermore, any animal which experienced irreversible VF were excluded from VPB quantification and arrhythmia time course analysis. All data sets, once collated were subject to analysis by the Grubbs' test to identify if there were any significant outliers from the rest of the data set. If a significant outlier was detected, it was excluded from further analysis and was not included for presentation in this chapter.

5.3.6 Statistical analyses

For analysis of haemodynamic variables, an unpaired T test was used to compare the pre- and post-injection MABP and HR data to determine any effect of bolus administrations of the drugs. A one-way ANOVA with Dunnett's post-hoc test was used to compare the pre- and post-occlusion values to determine the effect of ligation of a coronary artery on MABP and HR. A two-way ANOVA with Bonferroni post-hoc test was used to determine the effects of drug treatments on ischaemic MABP and HR. For the arrhythmia studies, a two-way ANOVA with Bonferroni post-hoc test was used to investigate the effect of drug treatment on number of VPBs in each of the quantified arrhythmia groups. The analysis of the drug treatment on distribution of arrhythmias in the 30 minutes ischaemic period was completed using a two-way ANOVA with Bonferroni post-hoc test. The Fisher's exact test was used to compare the effects of the drug interventions on the incidence on VF and in comparison of the onset on VF following drug treatment, a one-way ANOVA and Dunnett's post-hoc test was employed.

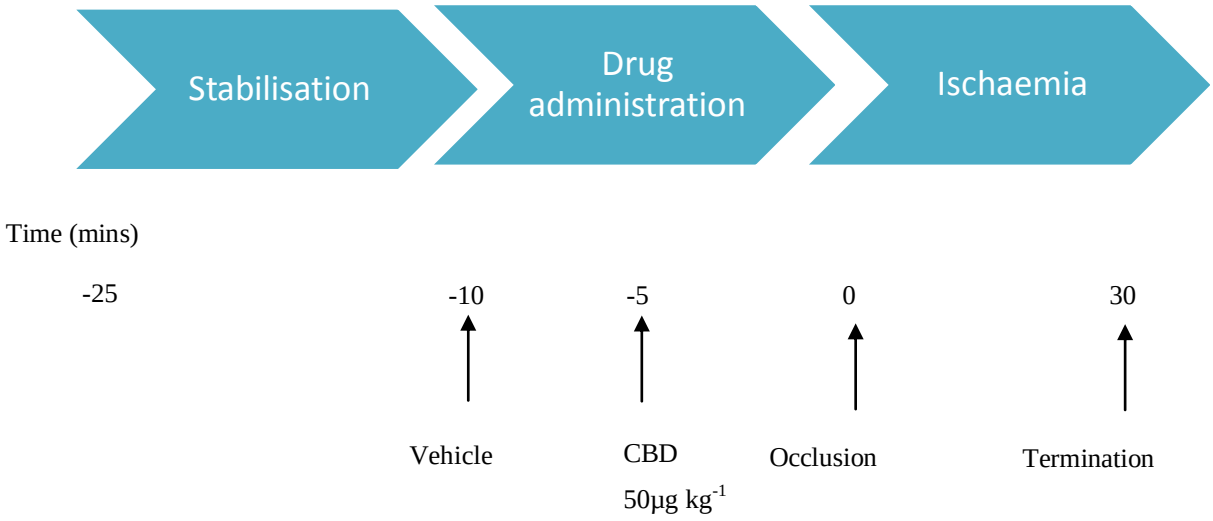

Figure 5.1 Experimental protocol for the characterisation of the outcome of CAO in anaesthetised rats in the presence of CBD (50µg kg^{-1}).

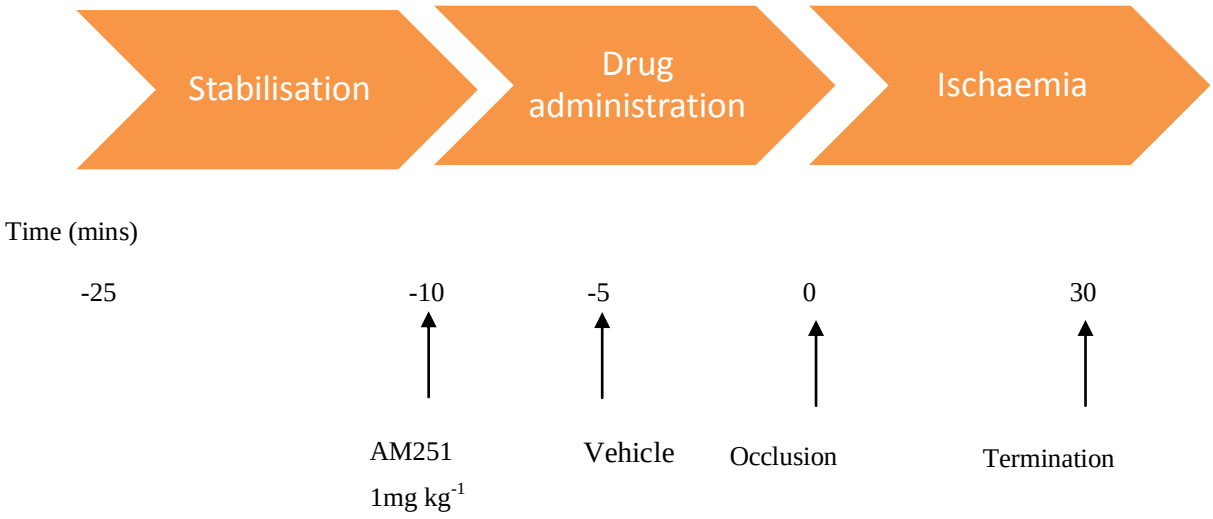

Figure 5.2 Experimental protocol for the characterisation of the outcome of CAO in anaesthetised rats in the presence of AM251 (1mg kg^{-1}).

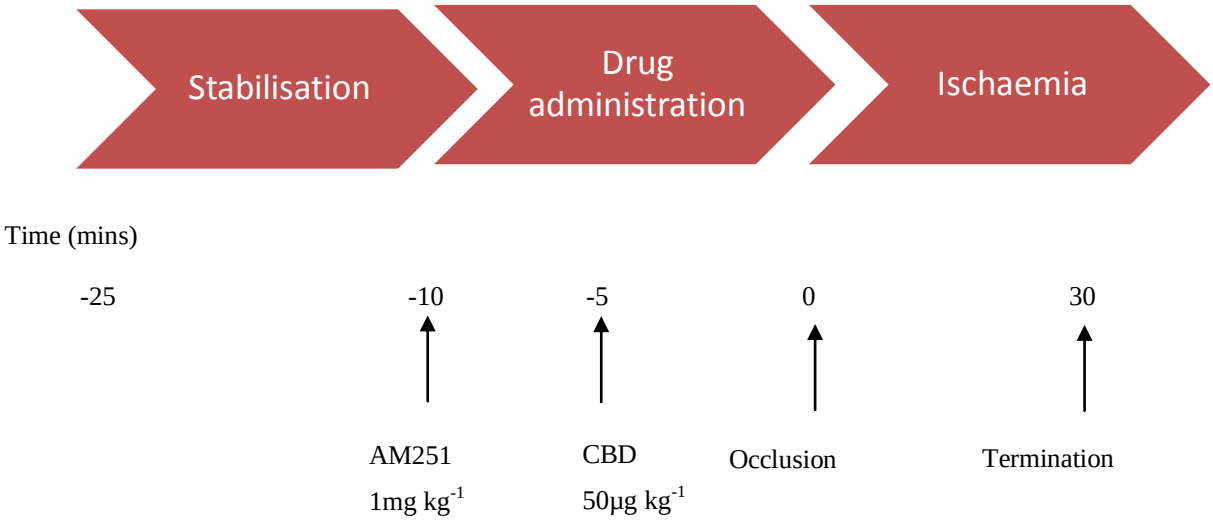

Figure 5.3 Experimental protocol for the characterisation of the outcome of CAO in anaesthetised rats in the presence of AM251 followed by CBD.

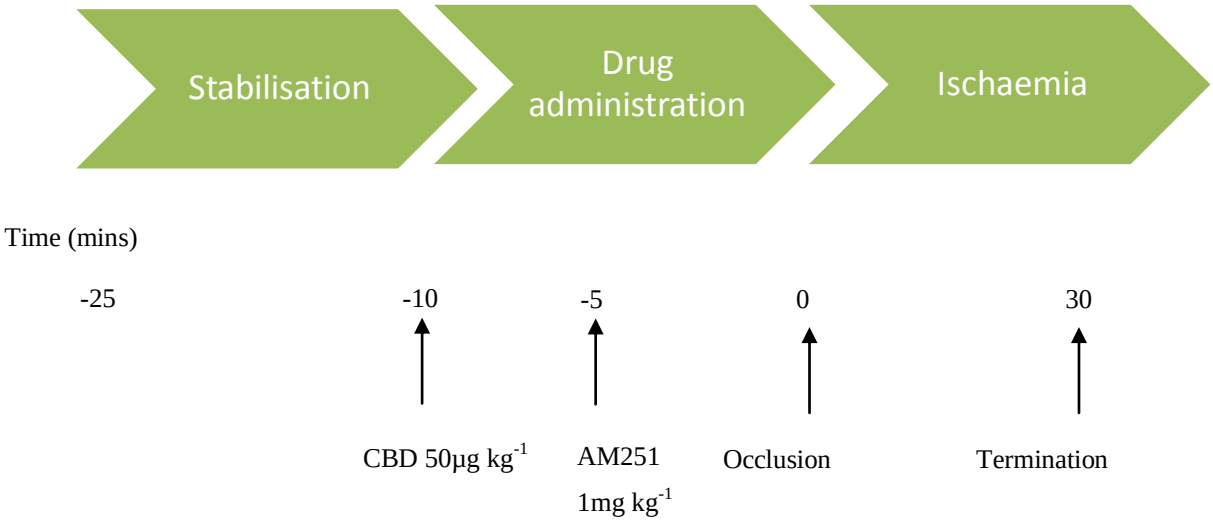

Figure 5.4 Experimental protocol for the characterisation of the outcome of CAO in anaesthetised rats in the presence of CBD followed by AM251.

5.4 Results

5.4.1 Exclusions

A total of 5 animals were excluded from analysis having met the exclusion criteria. 4 animals (3 from vehicle control and 1 from CBD (50µg kg^{-1})) were excluded after not presenting changes in the ECG that indicate successful induction of MI, 1 (CBD (50µg kg^{-1})) animal was excluded on the basis of low pre-occlusion BP.

Following quantification of VPBs for each experimental group, 4 animals (1 from AM251 (1mg kg^{-1}), 2 from CBD then AM241 and 1 from AM251 then CBD) were also excluded from VPB quantification and arrhythmia time course analysis, having developed irreversible VF.

5.4.2 Haemodynamic effect of pre-ischaemic treatment with CBD and AM251

Changes in MABP to bolus drug administration were also calculated in order to identify if any of the treatments significantly affected MABP in the 300 seconds post-administration (Figure 5.5 and Figure 5.6). CBD alone caused no change in MABP post-administration (Figure 5.5). Pre-treatment with AM251 caused a small but non-significant fall in MABP which recovered by bolus administration of CBD.

Bolus AM251 administration when given alone or when preceeded by CBD had no significant effect on MABP (Figure 5.6).

5.4.3 Haemodynamic effects of drug intervention and coronary artery occlusion

Occlusion of the LAD coronary artery induced an immediate fall in MABP of approximately 50% (115 ± 13 vs. 58 ± 13 mmHg; -25 and 1 minute, respectively; Figure 5.5 and Figure 5.6). Rats given CBD alone showed change (55% fall) in MABP similar to that observed in vehicle control treated animals following coronary occlusion (Figure 5.5). When CBD treatment was preceeded by AM251, the change in MABP upon CAO were similar to those seen with either CBD alone or vehicle control.

Post-ligation MABP recovery showed a trend toward improvement with AM251 both alone and when preceeded by CBD treatment (24% and 29%, respectively vs. 37%; % recovery between 1 and 30 minutes; Figure 5.6).

None of the treatment groups had any significant effect on HR changes observed in vehicle control animals (Figure 5.7 and Figure 5.8).

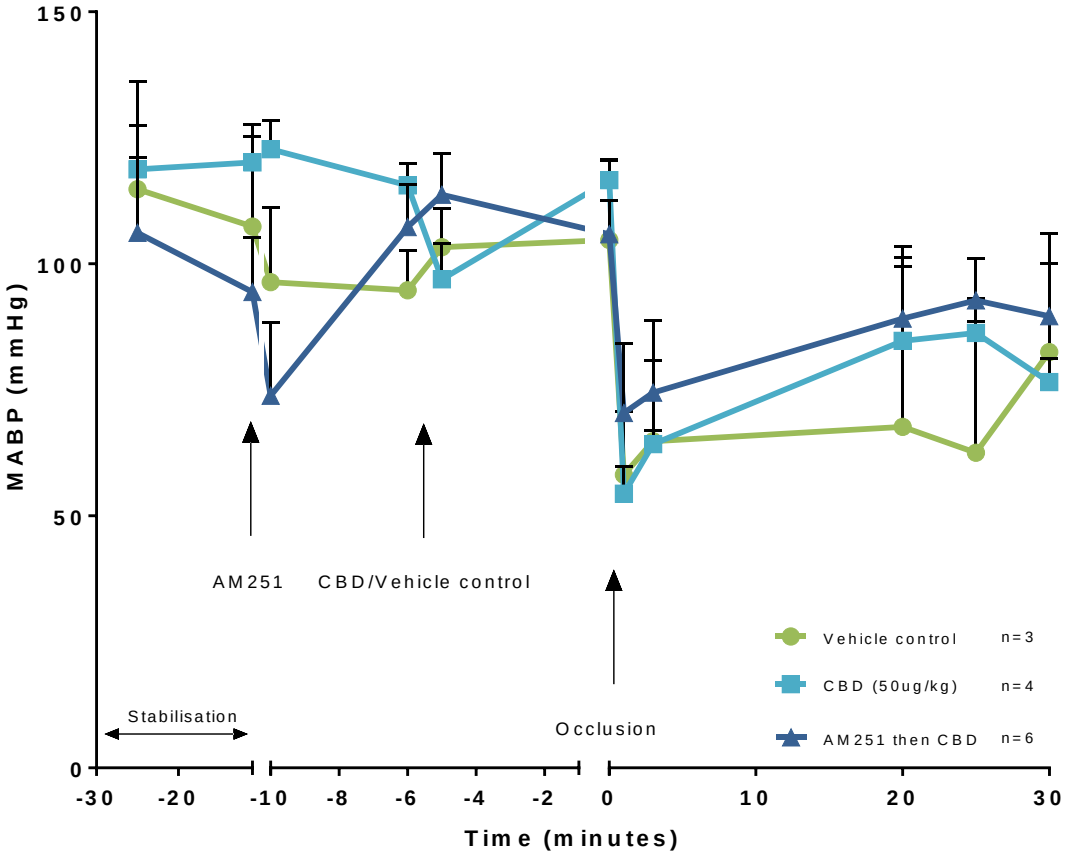

Figure 5.5 The effect of CBD, alone and in combination with AM251, on CAO-induced changes in MABP in anaesthetised rats. MABP was recorded prior throughout the protocol. Data is expressed as mean ± SEM (n= 3-6).

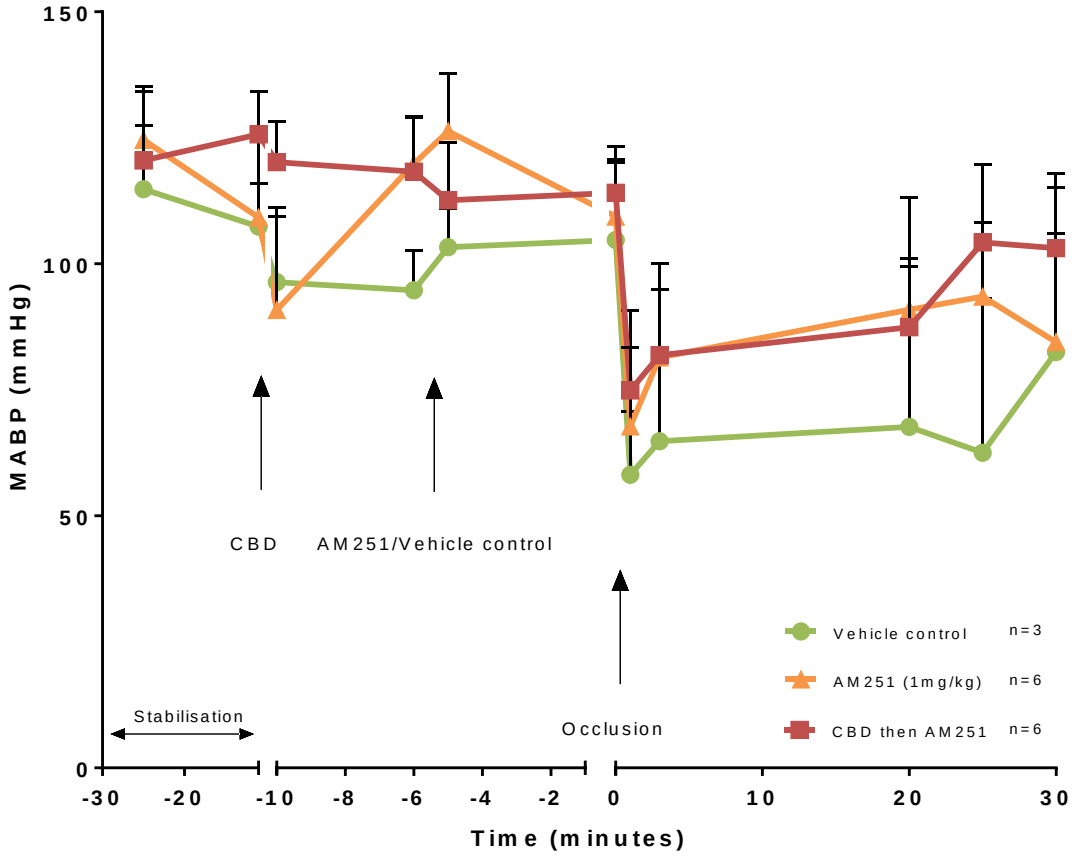

Figure 5.6 The effect of AM251, alone and in combination with CBD, on CAO-induced changes in MABP in anaesthetised rats. MABP was recorded throughout the protocol. Data is expressed as mean ± SEM (n= 3-6).

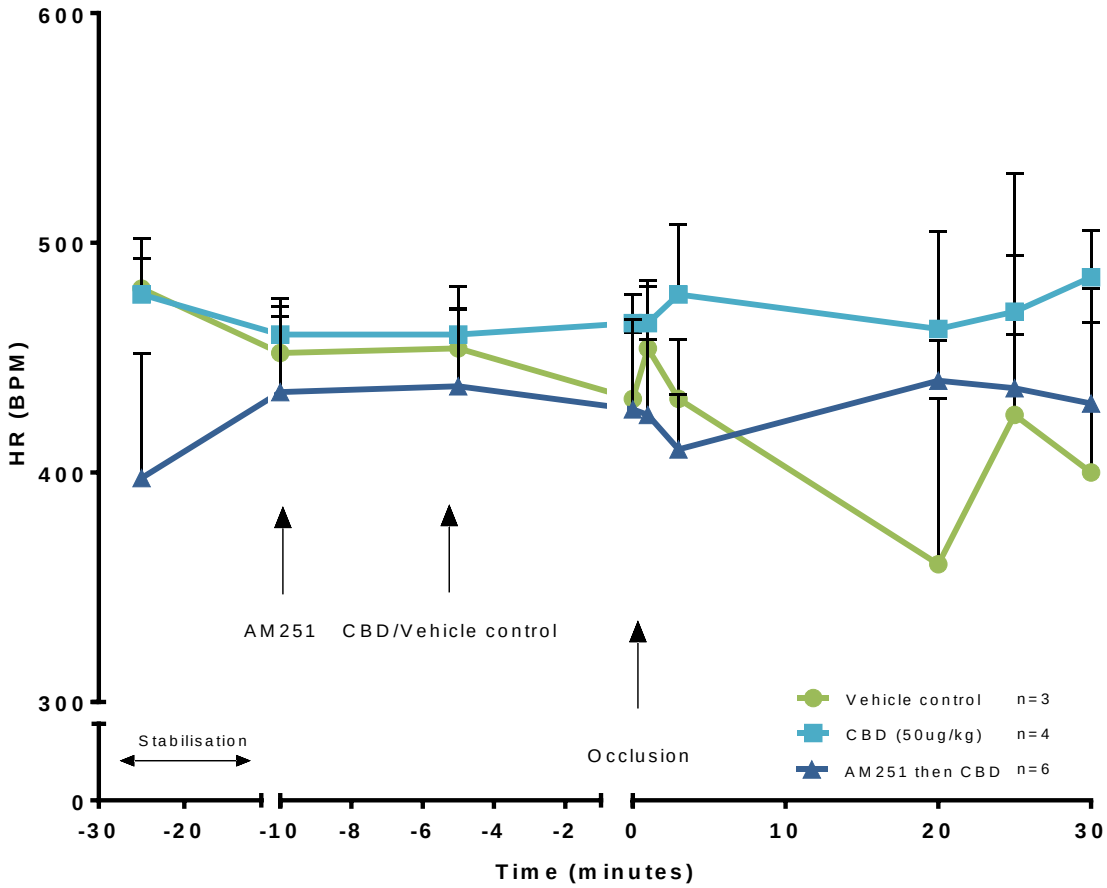

Figure 5.7 The effect of CBD, alone and in combination with AM251, on CAO-induced changes in HR in anaesthetised rats. HR was measured throughout the protocol. Data is expressed as mean ± SEM (n= 3-6).

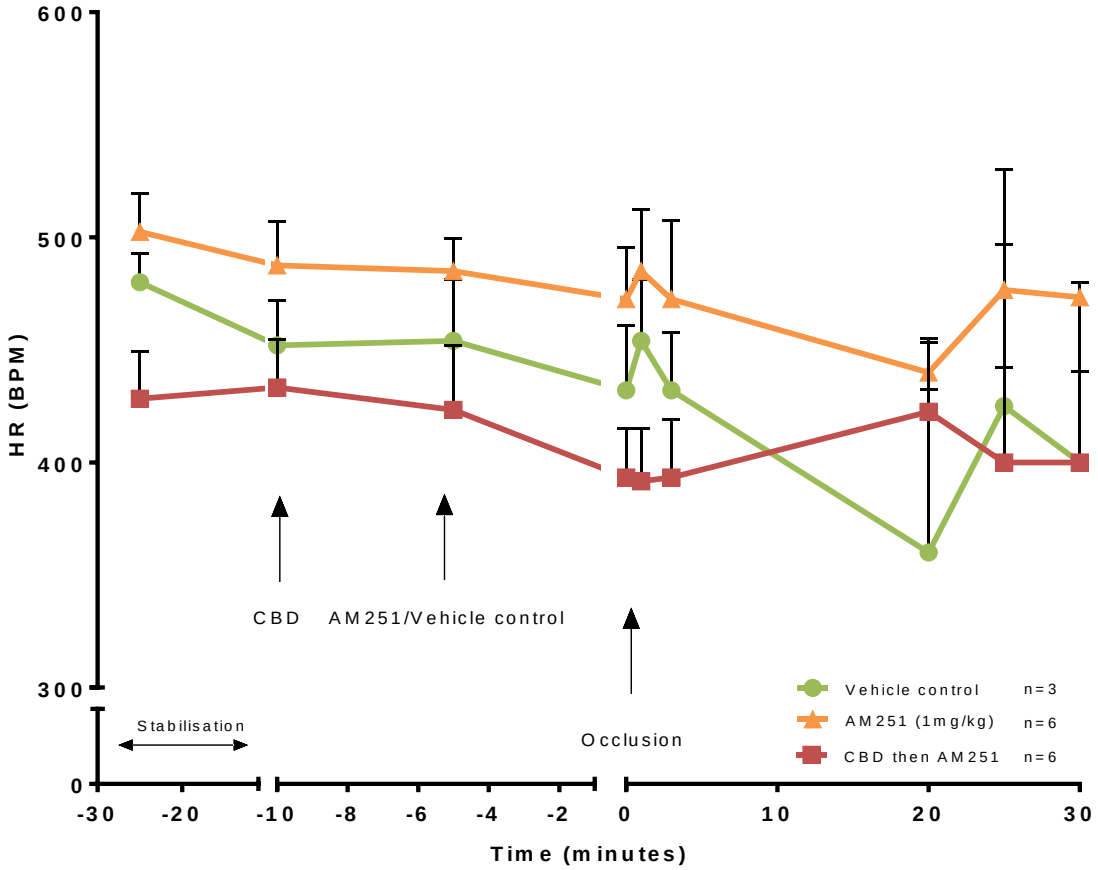

Figure 5.8 The effect of AM251, alone and in combination with CBD, on CAO-induced changes in HR in anaesthetised rats. HR was measured throughout the protocol. Data is expressed as mean ± SEM (n= 3-6).

5.4.4 Effect of CBD and AM251 on ischaemia-induced ventricular arrhythmias

Induction of experimental coronary artery occlusion induced all the forms of arrhythmic activity quantified in this study. This ventricular ectopic activity was present in all drug treatment groups (Figure 5.9 and Figure 5.10). In the vehicle control group, the majority of VPBs manifested as VT. None of the drug treatments had any significant effect on the number of arrhythmias which occurred as singles or salvos.

Administration of CBD alone prior to ischaemia attenuated the number of VPBs which occurred as VT (715 ± 205) when compared with the vehicle control (1441 ± 1049 VPBs; Figure 5.9) and consequently reduced the total number of VPBs (977 ± 229 vs. 1645 ± 974 VPBs; Figure 5.9) although this effect was not statistically significant. When bolus CBD administration was preceeded by AM251, there was a significant reduction in both the incidence of VT and the total number of VPBs when compared with the vehicle control (37 ± 16 (VT) and 133 ± 31 (total VPBs) vs. 1441 ± 1049 and 1645 ± 974; VPBs $P<0.05$ Figure 5.9).

Administration of AM251 alone reduced the number of arrhythmias which occurred as VT (253 ± 105 vs. 1441 ± 1049 VPBs) and total VPBs (496 ± 84 vs. 1645 ± 974; $P>0.05$; Figure 5.10) compared to vehicle control. When CBD was given 5 minutes prior to administration of AM251 a reduction in both VT and the total number of VPBs, similar to that seen with AM251 alone was observed (400 ± 220 (VT) and 351 ± 314 (total VPBs) vs. 1441 ± 1049 and 1645 ± 974; CBD then AM251; Figure 5.10).

5.4.5 Effect of CBD and AM251 on the time distribution of ischaemia-induced ventricular arrhythmias

In control animals, ventricular ectopic activity occurred predominantly in the 7-17 minutes post-ligation, with peak activity occurring at 11 minutes after cessation of regional blood flow (Figure 5.11). Arrhythmias were present in both phase Ia (2-10 minutes post-occlusion) and Ib (12-30 minutes post-occlusion) of the ischaemic period.

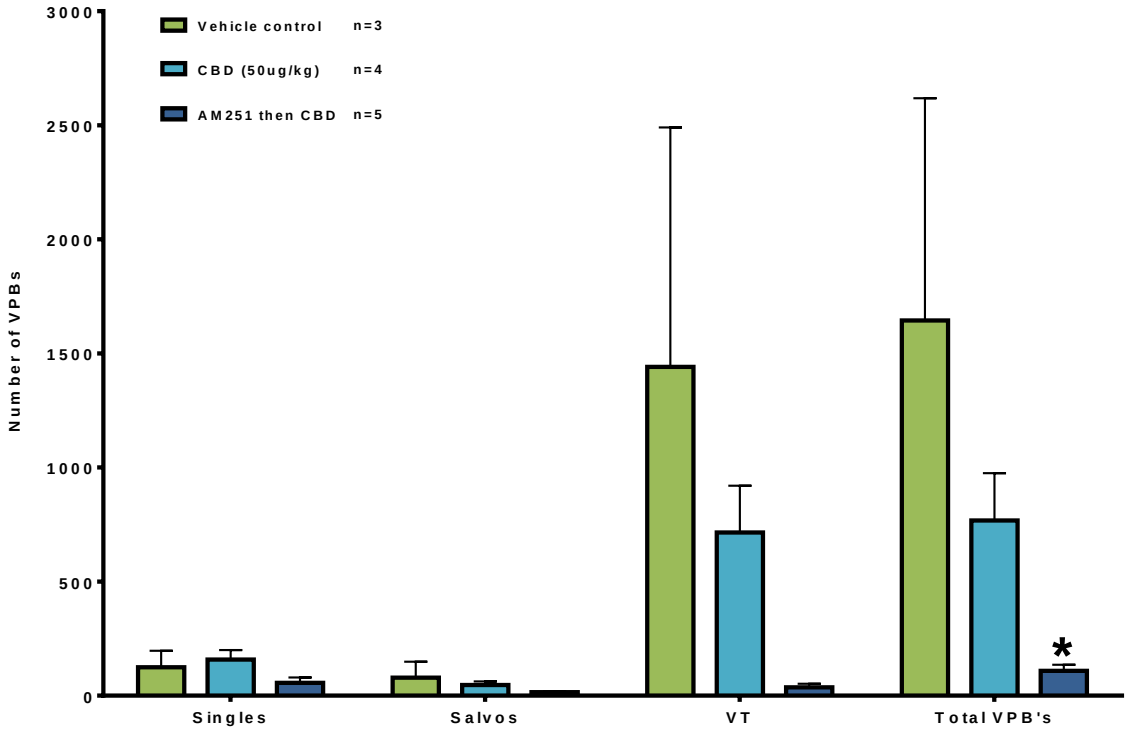

Figure 5.9 Effect of CBD (50µg kg^{-1}), separately and in combination with AM251 (1mg kg^{-1}), on ischaemia-induced ventricular arrhythmias. The incidence of each type of arrhythmia induced by ligation of a coronary artery was quantified and the data expressed as mean ± SEM (n=3-5). * indicates P<0.05 vs. vehicle control.

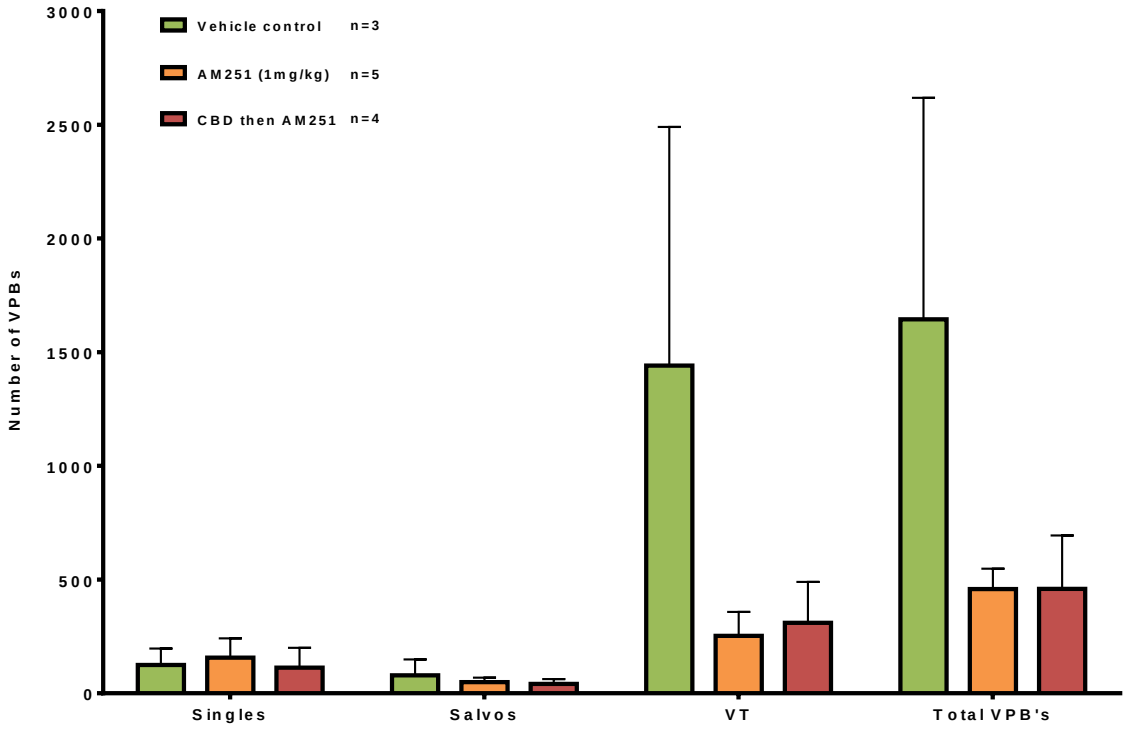

Figure 5.10 Effect of AM251 (1mg kg^{-1}), separately and in combination with CBD (50µg kg^{-1}), on ischaemia-induced ventricular arrhythmias. The incidence of each type of arrhythmia induced by ligation of a coronary artery was quantified and the data expressed as mean ± SEM (n=3-5).

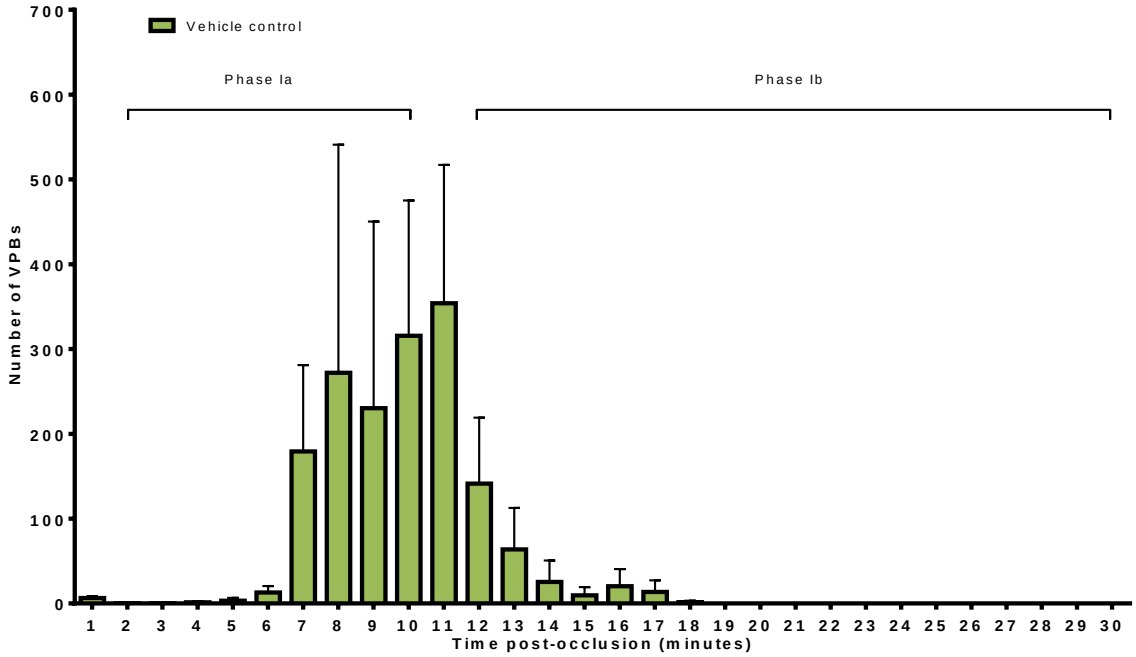

Figure 5.11 Time distribution of ischaemia-induced ventricular arrhythmias in control rats. The number of VPBs observed over 30 minutes of ischaemia. Data is expressed as number of VPBs (n=3).

Animals treated with CBD prior to ischaemia showed a similar time to onset of ventricular ectopic activity i.e. 5 minutes, and there was no marked difference in the distribution of arrhythmias within phase Ia and Ib when compared with the vehicle control treated animals (Figure 5.12) There was no significant differences in the overall arrhythmia distributions (Figure 5.12).

When CBD administration was preceded by AM251 (AM251 then CBD) VPB activity only presented in phase Ia (6-11 minutes post-occlusion), while in the vehicle control group, it occurred in both phases (Figure 5.13). The bi-phasic presentation of VPB activity which was observed with CBD alone was not observed when CBD treatment was preceded by AM251. Significant reductions in VPBs were seen at 8 (151 ± 69 vs. 272 ± 269 VPBs; $P<0.05$; Figure 5.13), 10 (10 ± 6 vs. 316 ± 159 VPBs; $P<0.001$; Figure 5.13) and 11 (68 ±45 vs. 354 ± 163 VPBs; $P<0.001$; Figure 5.13) minutes post-occlusion, when compared with vehicle control.

Animals treated with the CB_1 receptor antagonist and proposed GPR55 agonist, AM251, demonstrated a similar time to onset of ventricular ectopic activity as was seen with the vehicle treated animals (5 minutes; Figure 5.14). However, distribution of VPBs in this group was quite distinct from that observed in the vehicle control group as all VPBs occurred toward the latter stages of phase Ia and the beginning of phase Ib, in that a bi-phasic appearance of VPB activity was observed between 6-12 minutes (phase Ia) and the latter between 22-23 minutes (phase Ib) post-occlusion. Although, the period from onset to cessation of VPB activity in this group was lengthier than in control animals, there were significant reductions in the number of VPBs at 10 (10 ± 6 vs. 316 ± 159 VPBs; $P<0.001$) and 11 (68 ± 44 vs. 354 ± 163 VPBs; $P<0.005$; Figure 5.14) minutes post-occlusion (the phase when re-entry induced arrhythmias predominate) when compared to the control.

Interestingly, co-administration of both compounds in the reverse order (CBD then AM251) did not provide as intense an additive effect, as was observed with AM251 then CBD (Figure 5.15). In this drug treatment group, the onset of VPBs was slightly later than that observed in control animals (6 minutes vs. 5 minutes). VPB activity was present in both phases in this group. That being said, there were significant reductions in the number of VPBs at; 10 (63 ± 27 vs. 316 ± 159 VPBs; CBD then AM251 vs. vehicle control; $P<0.05$; Figure 5.15) and, 11 minutes (17 ± 17 vs. 354 ± 163 VPBs; CBD then AM251 vs. vehicle control; $P<0.001$; Figure 5.15). Compared with AM251 alone, CBD then AM251 didn't potentiate the reduction seen with AM251 alone, but the significant reductions in VPBs observed at 10 and 11 minutes post-occlusion remained.

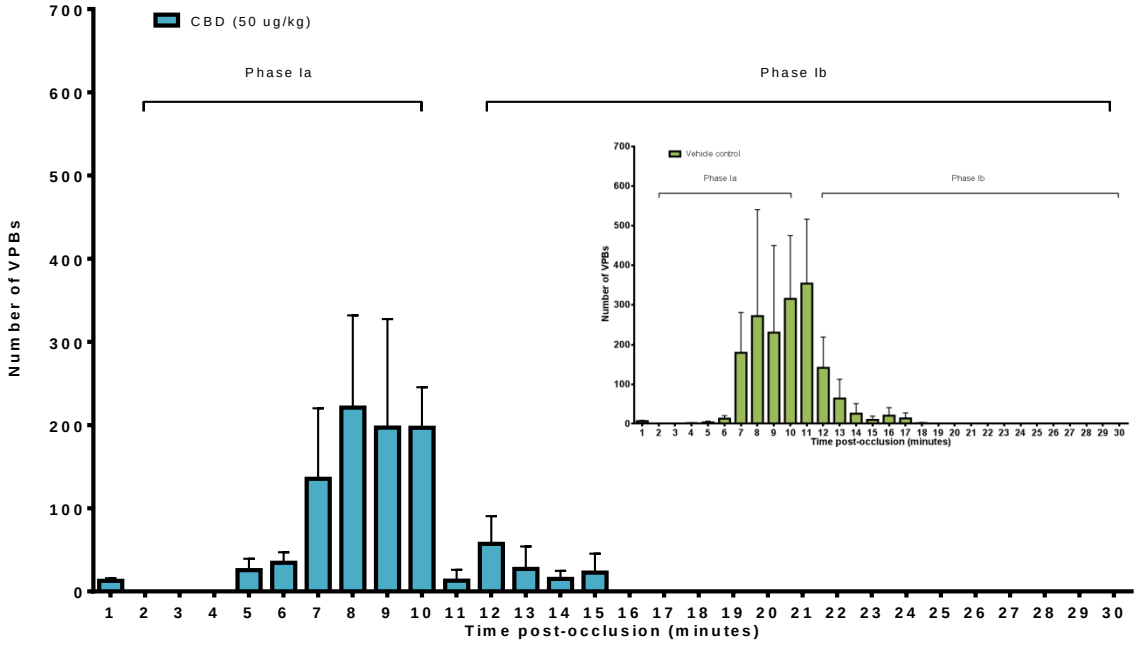

Figure 5.12 Effect of CBD (50µg kg^{-1}) on the distribution of ischaemia-induced ventricular arrhythmias. The number of VPBs observed over 30 minutes of ischaemia. Data is expressed as number of VPBs (n=4). * indicates P<0.05 vs. corresponding time point in vehicle control.

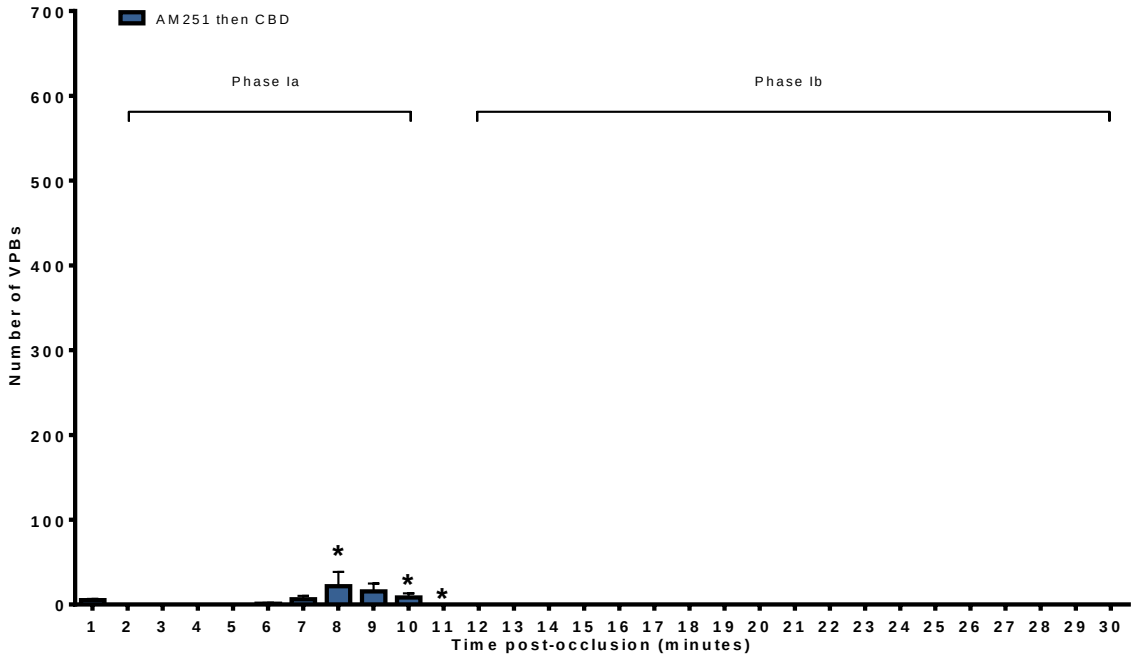

Figure 5.13 Effect of AM251 followed CBD on the distribution of ischaemia-induced ventricular arrhythmias. The number of VPBs observed over 30 minutes of ischaemia. Data is expressed as number of VPBs (n=5).

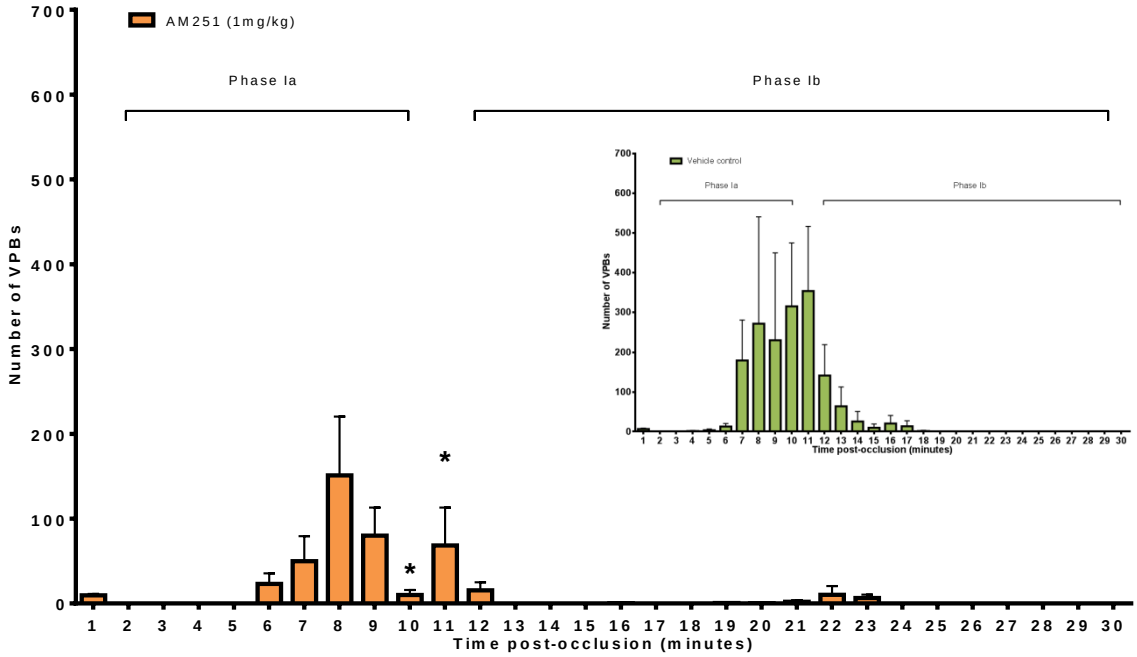

Figure 5.14 Effect of AM251 (1mg kg^{-1}) on the distribution of ischaemia-induced ventricular arrhythmias. The number of VPBs observed over 30 minutes of ischaemia. Data is expressed as number of VPBs (n=5). * indicates P<0.05 vs. corresponding time point in vehicle control.

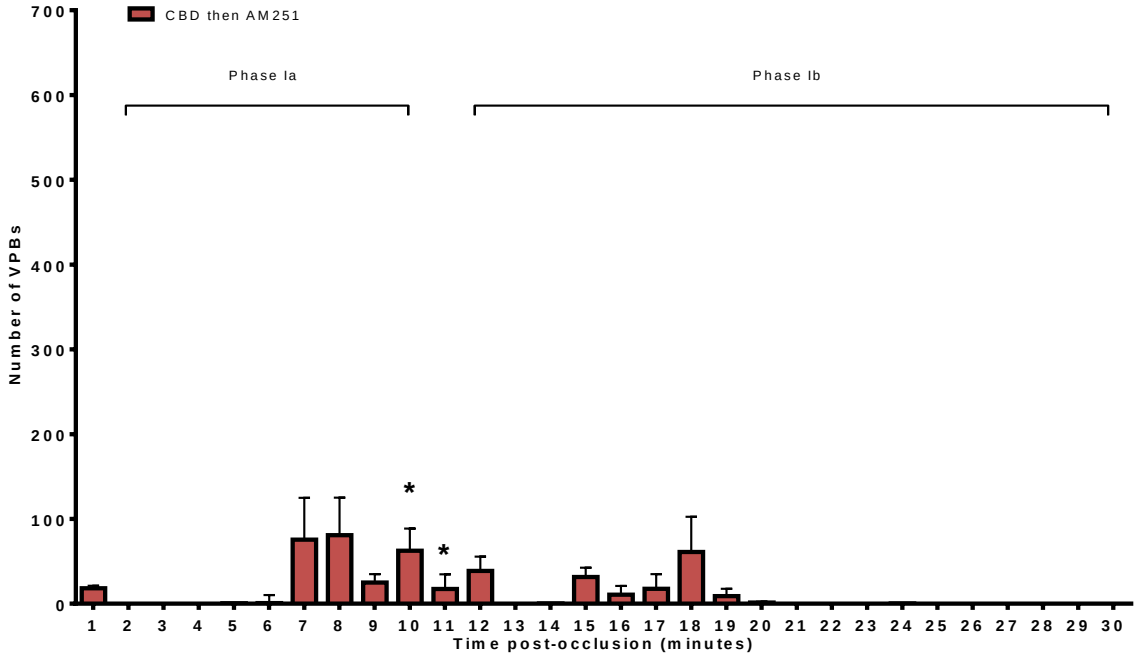

Figure 5.15 Effect of CBD followed by AM251 on the distribution of ischaemia-induced ventricular arrhythmias. The number of VPBs observed over 30 minutes of ischaemia. Data is expressed as number of VPBs (n=4).

5.4.6 Effect of CBD and AM251 on the incidence of ventricular fibrillation following coronary artery ligation

Occlusion of the LAD coronary artery in the vehicle control group, induced a total incidence of VF of 77%, of which 60% was reversible and 17% resulted in mortality (Figure 5.16 and 5.17). Pre-ischaemic administration of CBD abrogated the incidence of irreversible VF (0 vs. 17%; CBD and vehicle control, respectively) and also, brought about a reduction in the incidence of reversible VF. AM251 pre-treatment also induced a reduction in reversible VF (40 vs. 60%; AM251 and vehicle control, respectively) although, irreversible VF in this group was still present, total VF was reduced to 50%. Co-administration of AM251 then CBD induced a significant reduction in total VF (17 vs. 77%; AM251 then CBD vs. vehicle control; $P<0.05$) and elimination of reversible VF (0 vs. 60%) compared with vehicle control. No such effect was observed with CBD followed by AM251; in contrast, in this drug treatment group there was exacerbation of all forms of VF. Overall, mortality was reduced by all drug treatments, with the exception of the co-administration of CBD followed by AM251, in which, mortality showed a trend toward being increased.

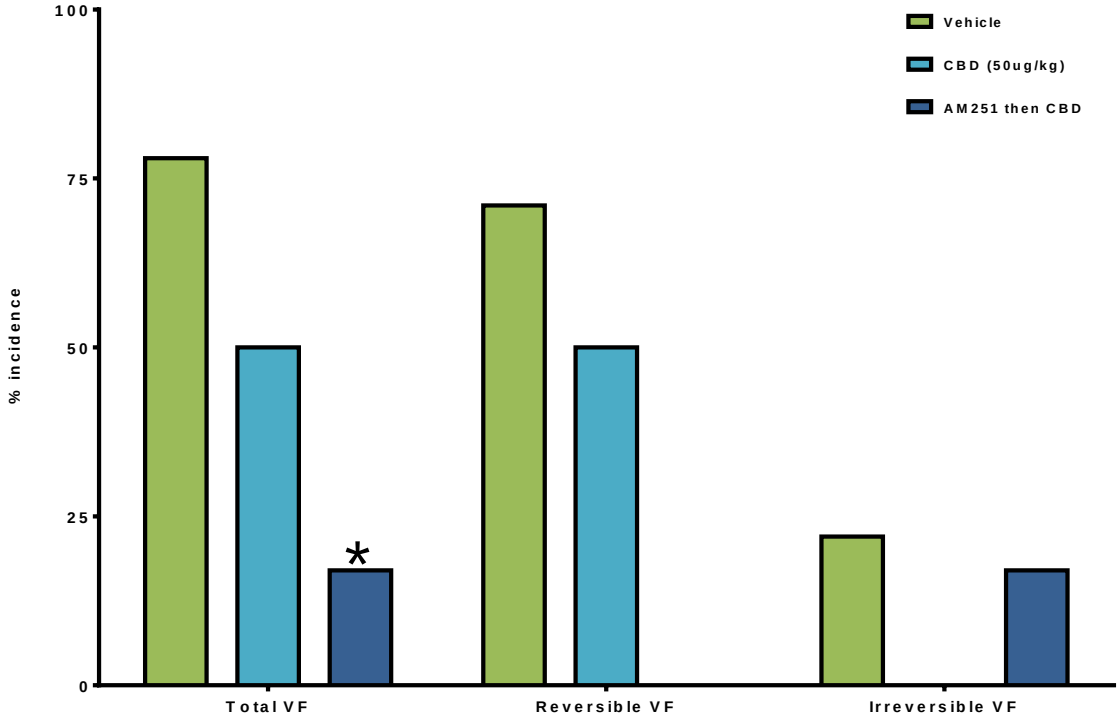

Figure 5.16 Effect of CBD with and without AM251 pre-treatment, on the incidence of VF following ligation of a coronary artery. The incidence of total, irreversible and reversible VF were quantified and the data expressed as % incidence (n=1-4). * indicates P<0.05 vs. vehicle control.

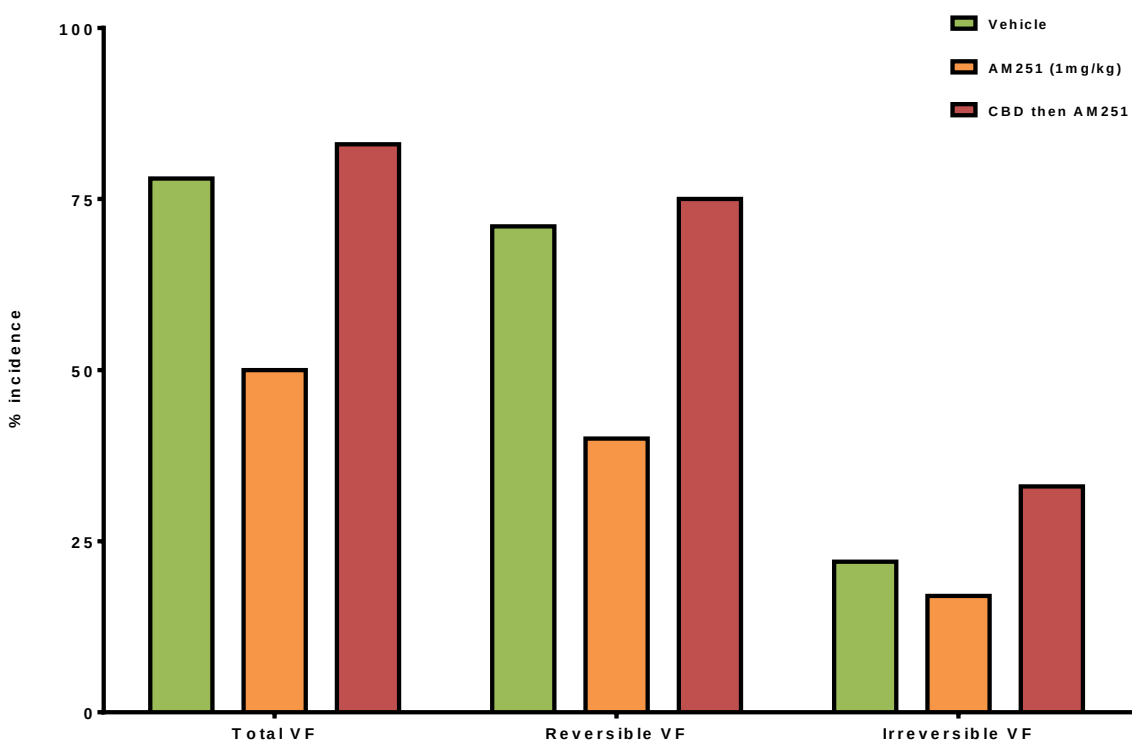

Figure 5.17 Effect of CBD and AM251, together and separately, on the incidence of VF following ligation of a coronary artery. The incidence of total, irreversible and reversible VF were quantified and the data expressed as % incidence (n=1-4).

5.4.7 Effect of CBD and AM251 on ventricular fibrillation following coronary artery ligation

VF was present in all drug treatment groups (Table 5.2). CBD alone reduced the time to onset of VF compared with vehicle control (480 ± 36 vs. 696 ± 54; CBD vs. vehicle control, respectively). Pre-treatment with AM251 did not affect CBD-mediated changes in time to VF onset. AM251 alone significantly reduced time to VF onset (437 ± 102 vs. 696 ± 54; AM251 vs. vehicle control, respectively; $P<0.05$). This AM251-mediated reduction in time to VF onset was potentiated by pre-treatment with CBD (8 vs. 696 ± 54; CBD then AM251 vs. vehicle control, respectively) although, VF was only recorded in 1 rat in this group and thus statistical significance could not be ascertained.

Time to onset (secs)	Vehicle control n=3	CBD (50µg kg^{-1}) n=3	AM251 (1mg kg^{-1}) n=3	CBD then AM251 n=1	AM251 then CBD n=4
VF	696 ± 54	480 ± 36	437 ± 102*	8	408 ± 138

Table 5.2 Effect of CBD (50µg kg^{-1}) and AM251 (1mg kg^{-1}), separately and in combination, on the onset of ischaemia-induced VF. The onset of VF was quantified from ECG recordings following ligation of a coronary artery. * indicates P<0.05 vs. vehicle control. Data is expressed as mean ± SEM (n=1-4).

5.4.8 Identification of GPR55 receptor in rodent myocardium

Rodent myocardium stained by IHC with a specific antibody directed against GPR55 showed positive staining. In sham tissue this staining was observed in cardiomyocytes, endothelial cells and also in inflammatory cells (Figure 5.18). In the sections from hearts subject to MI there is evident damage to the myocardium as it appears the cardiomyocytes had become separated from one another. However, staining for GPR55 was still evident in the cardiomyocytes and endothelial cells of the lumens of blood vessels (Figure 5.19). GPR55 staining in hearts subject to pre-ischaemic bolus CBD treatment showed more diffuse positive staining in cardiomyocytes, however, endothelial cells were stained to a similar degree as was observed in sham and ischaemic heart sections (Figure 5.20).

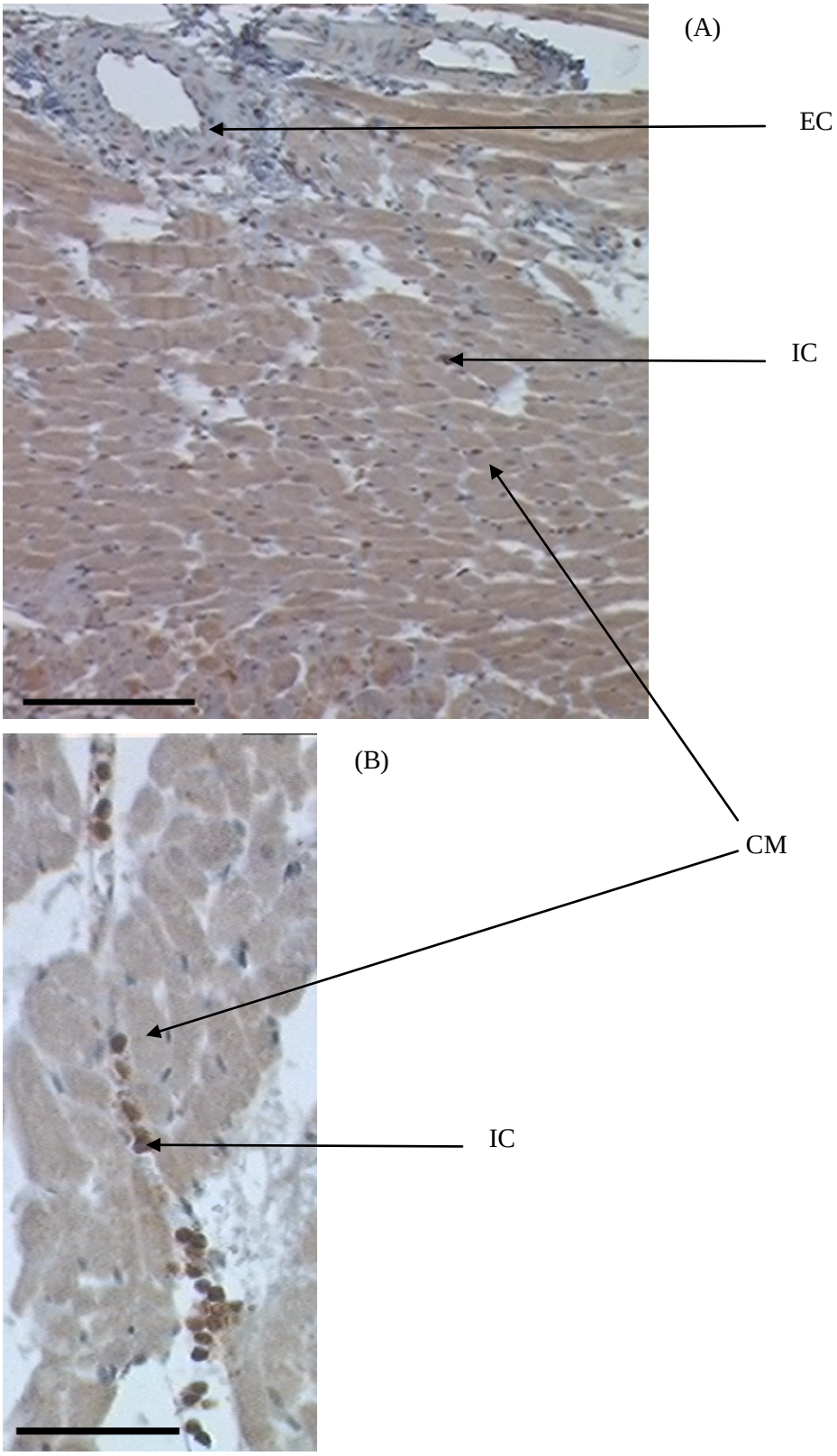

Figure 5.18 IHC staining for GPR55. Light micrographs of sections of rat heart tissue at (A) x100 and (B) x200 magnifications and an antibody concentration of 1:750. Image (A) shows positive staining for GPR55 in the cardiomyocytes (CM) and endothelial cells (EC). Image (B) shows, at a higher magnification, positive staining in inflammatory cells (IC). Scale bar is 170µm.

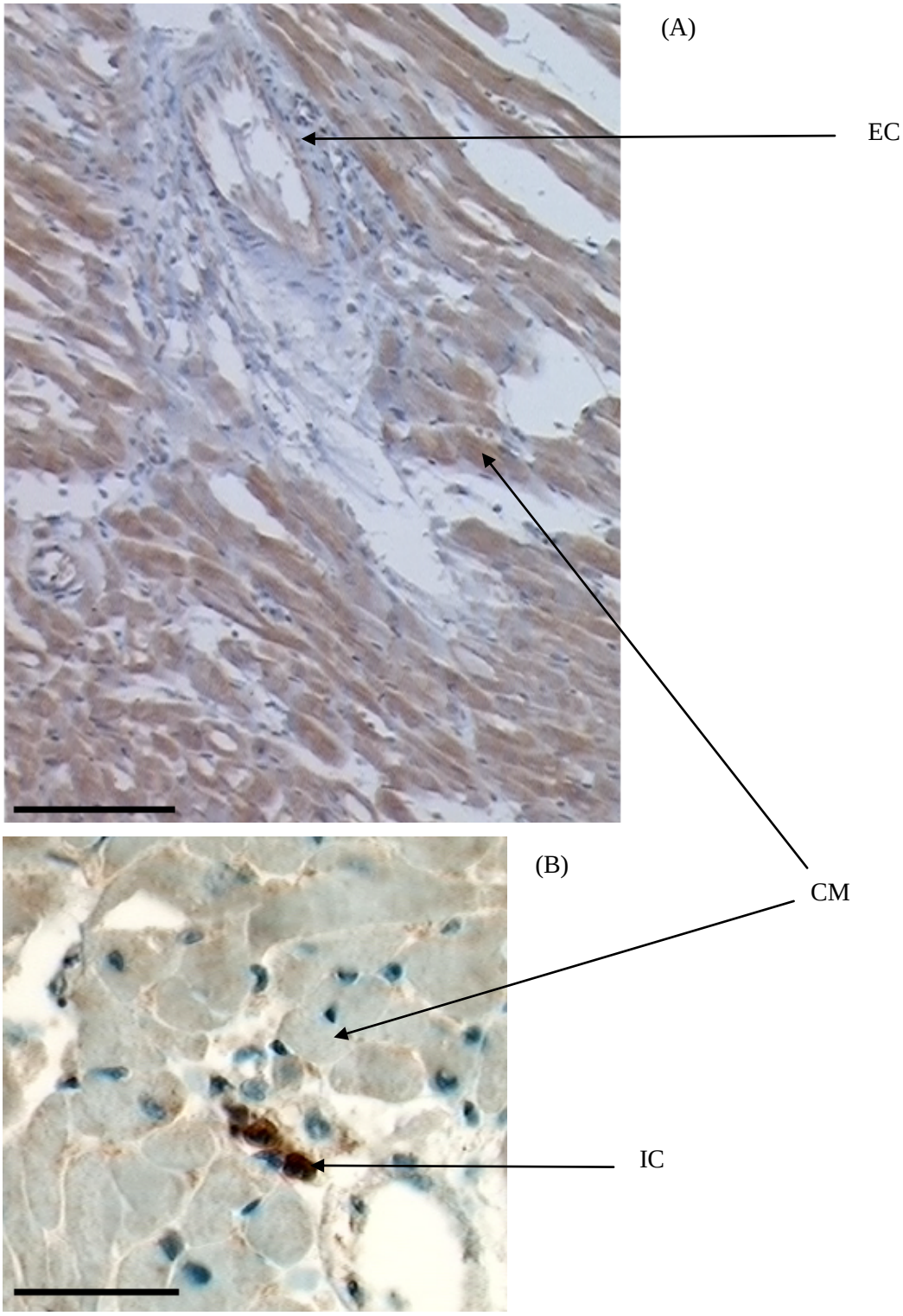

Figure 5.19 IHC staining for GPR55 in ischaemic heart tissue. Light micrographs at (A) x100 and (B) x400 magnifications. Image (A) shows positive staining for GPR55 in the cardiomyocytes (CM) and endothelial cells (EC). Image (B) shows positive staining for GPR55 in the inflammatory cells (IC). Scale bar is 170µm.

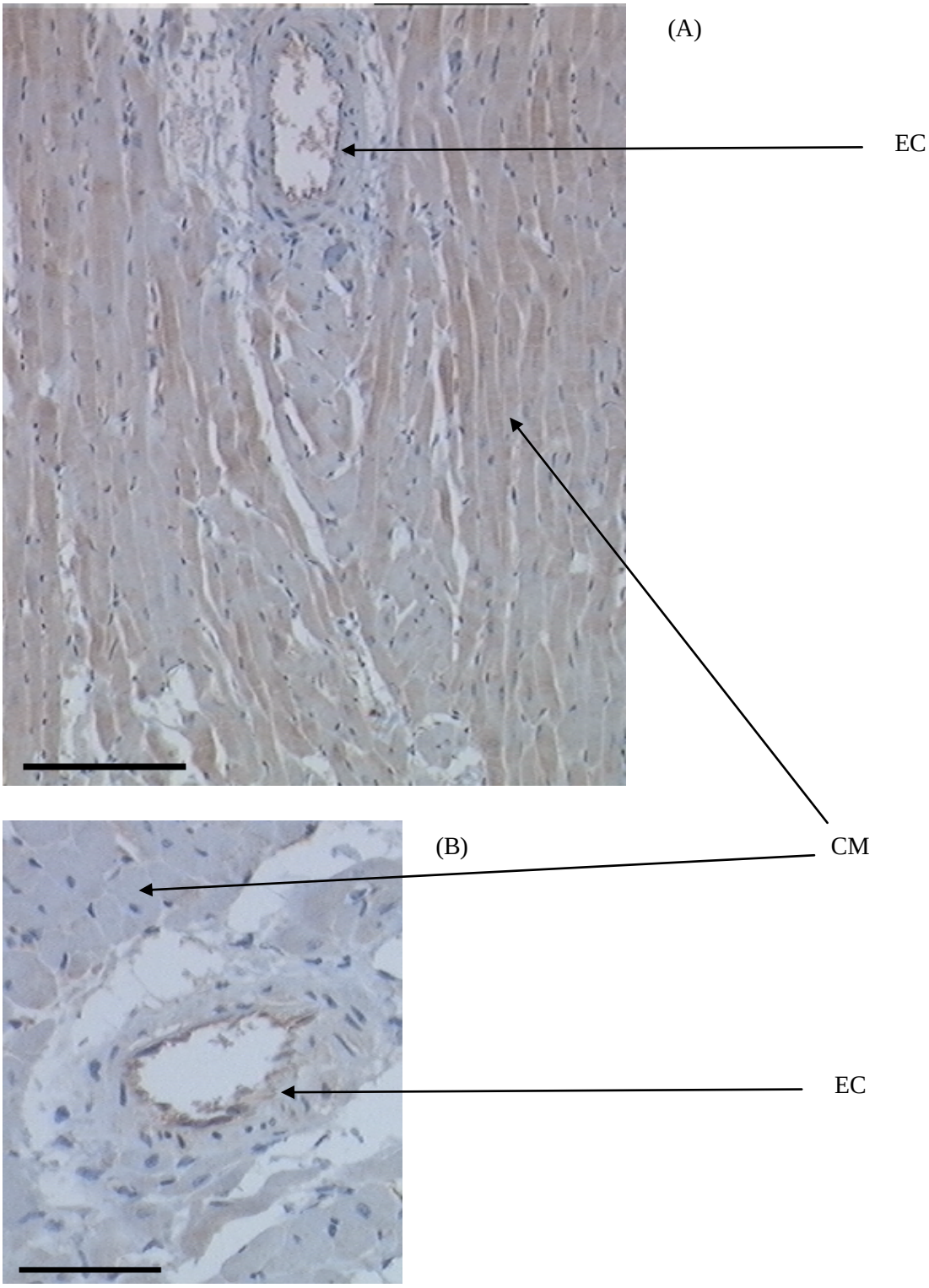

Figure 5.20 IHC staining for GPR55 in CBD-treated ischaemic heart tissue. Light micrographs at (A) x100 and (B) x200 magnifications. Image (A) shows diffuse positive staining for GPR55 in cardiomyocytes (CM) and positive staining in endothelial cells (EC) which is shown in detail in image (B). Scale bar is 170µm.

5.5 Discussion

5.5.1 Effects of CBD on ischaemia-induced ventricular arrhythmias, MABP and HR

In this study bolus CBD administration was shown to have no apparent effect on MABP or HR before or during CAO.

The phytocannabinoid CBD, when administered prior to the onset of a period of MI, possesses the capacity to reduce ischaemia-induced ventricular arrhythmias by reducing VT, although this was not statistically significant. The anti-arrhythmic effect was principally due to a reduction in phase Ia arrhythmias. VF incidence was unaffected by treatment with CBD. These findings are consistent with those previously reported (Walsh *et al.*, 2010). In the presence of AM251, data in this study demonstrated that CBD significantly reduced VPBs and abolished arrhythmias in phase Ib. Moreover, there was a marked reduction in VF incidence.

In hearts subjected to coronary artery occlusion, ventricular action potentials undergo a biphasic change which includes an initial lengthening, followed by a more prolonged shortening. In addition the refractory period of the ventricular action potential shortens in line with the shortening of action potential duration (Brooks *et al.*, 1960; Janse and Wit, 1989). These changes in refractoriness, coupled with the slowed conduction in the ischaemic myocardium, provide a suitable milieu for the facilitation of reentry (Osadchii, 2010). It has been proposed that reentry is the dominant mechanism responsible for the initiation of ventricular tachycardia in the early stages of myocardial infarction (Boineau and Cox, 1973; Janse and Wit, 1989). During MI, activation of delayed rectifier potassium current (I_{k1}) prevents excess activation of myocytes in the ischaemic zone and therefore, may prevent the generation of arrhythmias. I_{k1} has been shown to be induced by activation of the CB_1 receptor (Felder *et al.*, 1995) while CBD has been reported to inhibit both the rapid and slow activating facets of I_{k1} in isolated ventricular myocytes (Mamas and Terrar., 1998). In light of the findings in Chapter 4 that, at the dose used in the present study, CBD appears to exhibit some CB_1 antagonist activity; this could conceivably represent the mechanism of its anti-arrhythmic effects. However, since CBD does not prolong QT interval prior to ischemia or potentiate ischaemia-induced QT interval prolongation, it seems unlikely that this is the mechanism by which CBD is anti-arrhythmic (Walsh *et al.*, 2010). In the presence of AM251, the effects of CBD could not be reversed but instead enhanced its effects. It demonstrated that AM251 is itself anti-arrhythmic. This may also infer that the CB_1 receptor is not

involved in the mediation of the anti-arrhythmic effect of CBD. Moreover, the failure of AM251 (CB_1 antagonist) to reverse the effects of CBD similarly points to a lack of involvement of CB_1.
An alternative explanation could be a direct action of CBD at LTCCs, since compounds which block LTCCs, aid in the protraction of conduction and refractoriness in the myocardium (Harrison, 1985) and are used clinically as a first-line treatment for prevention of fatal arrhythmias following myocardial infarction (British National Formulary, 2013). Indeed CBD has been observed to slow intracellular Ca^{2+} accumulation by inhibition of LTCCs (Mamas and Terrar, 1998). Further investigation of the effect of CBD on intracellular Ca^{2+} homeostasis and excitation-induced Ca^{2+} mobilisation are discussed in chapter 6.

The complex pharmacology of CBD is such that involvement of non-classical cannabinoid receptors in the mediation of cardioprotection must be given due consideration. Indeed, evidence from our laboratory shows that CBD is unable to prevent the hypotensive effect of the CB_1 receptor agonist ACEA *in vivo*, suggesting that CBD is not operating as an antagonist at the CB_1 receptor. It is now well established that CBD acts as an antagonist of the de-orphanised GPCR, GPR55. Daly et al. (2010) used a novel, putative, fluorescent form of AM251 (also known to act as a GPR55 ligand) to identify the localisation of a cannabinoid-type receptor purported to be GPR55 in the small arteries of the tail and mesentery of C57Bl/6J mice and Wistar rats. Positive staining for GPR55 was localised to the endothelium of rat tail arteries and around the nucleus of Schwann cells. In addition, histological analysis of GPR55 in the myocardium and vasculature by our laboratory has shown that GPR55 is located in both the heart and the vasculature. Furthermore, GPR55 receptor localisation is observed to be concordant with that of the classical cannabinoid receptors suggesting that GPR55 may be involved in the mediation of the cardioprotective effects of CBD and that there may be an element of dimerisation or synergism between cannabinoid receptors and GPR55 (Ryberg *et al*., 2007; Staton *et al*., 2008; Daly *et al*., 2010).

Another consideration as to the mechanism of CBD-induced cardioprotection is the involvement of the purine nucleoside, adenosine. Adenosine is widely distributed within the body but interestingly, the extracellular concentrations of it are significantly amplified during periods of ischaemia (Schulte and Fredholm, 2003; Cohen and Downey, 2008). What is more, adenosine is profoundly anti-arrhythmic (Wainwright & Parratt, 1988), has been found to initiate ischaemic preconditioning by direct coupling to protein kinase C (PKC), and has been used in the treatment of supraventricular arrhythmia (Liu *et al*., 1991; Cohen and Downey, 2008). CBD has been shown to transactivate adenosine receptors (Wainwright and Parratt, 1993) and thus enhancement of adenosine signalling which is known to anti-arrhythmic may play a role in its cardioprotective effects.

5.5.2 Effects of AM251 on ischaemia-induced ventricular arrhythmias

This study has demonstrated for the first time that the CB_1 receptor antagonist AM251 has significant anti-arrhythmic effects when administered before the onset of ischaemia. This is in contrast to the findings by Hajrasouliha *et al*. (2008), who showed that AM251 provided no anti-arrhythmic benefit in a rat study whose experimental design was comparable with the present study. However, the VT count in the control group in the Hajrasouliha et al., study was much lower than that observed in control animals in the present study and similar to that observed in the AM251 treatment group and. The control animals in our study showed VT and total VPB incidences comparable with those in existing literature (Johnston *et al*., 1983; Crockett *et al*., 2000; Canyon and Dobson, 2004; Kloner, Dow and Bhandari, 2011). Since the Hajrasouliha et al. study had a lower starting arrhythmia counts, a reduction in arrhythmias by AM251 was likely compromised.

While the rationale for the use of AM251 in this present study was fundamentally to further investigate the pharmacology of CBD's anti-arrhythmic effects and to determine whether or not CBD was acting at CB_1 receptors, the fact that AM251 itself was anti-arrhythmic merits some discussion. The mechanisms involved in the suppression of arrhythmias by AM251 may be in part due to blockade of the CB_1 receptor, thereby allowing cardioprotective endocannabinoids (upregulated following ischaemia) to preferentially act at the CB_2 receptor, since the CB_2 receptor has been well characterised to confer cardioprotection (Krylatov *et al*., 2001; Zhang *et al*., 2008; Lim *et al*., 2009). Indeed there is evidence that CB_1 receptor expression is downregulated in the presence of increased endogenous AEA levels (e.g. in MI), representing a possible endogenous response to protect against ischaemia-induced injury/electrophysiological disturbances (Mukhopadhyay *et al*., 2007; Nucci *et al*., 2007; Cappellano *et al*., 2013). The involvement of both cannabinoid receptors in the induction of resistance to ischaemia and prevention of arrhythmias has been shown previously with the $CB_{1/2}$ receptor agonist, HU-210 in a rat model of global ischaemia in the isolated heart. The addition of HU-210 *in vitro* was associated with a reduction in the biomarker creatine phosphokinase in the coronary effluent and a reduction in left ventricular developed pressure (Krylatov *et al*., 2001; Lasukova *et al*., 2008). In addition, it has previously been proposed that CBD-mediated increased in $[Ca^{2+}]_i$ may stimulate endocannabinoid release, which may in turn activate the cannabinoid receptors ($CB_{1/2}$; Drysdale *et al.*, 2006). That being said, more recent data questions the likelihood that endocannabinoids protect against ischaemia-induced ventricular arrhythmias, since in a rat an isolated heart model of acute ischaemia, AEA and 2-AG both failed to reduce the incidence reperfusion-induced VF (Andrag and Curtis, 2013). In addition, they showed that in the late stage of acute regional ischaemia (30-60 minutes), AM251 exacerbated the incidence and duration of episodes of VF.

Lim et al. (2009) identified that a different CB_1 receptor antagonist, rimonabant, reduces infarct size. They hypothesised that the beneficial effects of rimonabant were associated with increased levels of the infarct sparing, anti-fibrillatory, fat-derived protein, adiponectin (Li and Liu, 2009; Kourliouros *et al*., 2011). However, although adiponectin has been shown to be synthesised in cardiomyocytes (Ishikawa *et al*., 2003; Takahashi *et al*., 2005) and rimonabant to increase its levels in adipose tissue (Bensaid *et al*., 2003), adiponectin levels in blood serum and heart tissue were only marginally different from those observed in control animals (Lim *et al*., 2009). Furthermore, there was no significant correlation between circulating adiponectin concentrations and the incidence of atrial fibrillation (Rienstra *et al*., 2012). Since it is unclear whether AM251 affects adiponectin synthesis, whether or not adiponectin plays a part in the anti-arrhythmic mechanism of AM251 remains to be determined.

An interesting finding in the present study was that AM251-had a marked suppressant effect on phase 1b arrhythmias, which are largely due to catecholamine flux. Existing literature has shown that CB_1 receptor agonists reduce, rather than increase, electrically evoked (and thus noradrenaline-dependent) contractions in nerve-muscle preparations (Schlicker and Kathmann, 2001). This would therefore rule out a mechanism for AM251 involving a reduction in noradrenaline (NA) release. Further evidence against a role for an effect of AM251 on NA release comes from the work of Kurihara et al. (2001), which showed that in rat heart sympathetic neurons, AM251 reversed the HU-210-induced inhibition of NA release. What is more, AM251 did not affect basal NA release, highlighting that the CB_1 receptor is not tonically activated by endogenous ligands. Taken together, this suggests that the anti-arrhythmic effects of AM251 observed in our study are not mediated by reductions in NA release from sympathetic neurons in the heart. That being said, although AM251 may not affect NA release from nerve terminals, literature exists to suggest that AM251 has the capacity to reduce NA synthesis. Catecholamine synthesis is rate-limited by a step early in the cascade of synthesis, which is catalysed by tyrosine hydroxylase. AM251 has been shown to reduce tyrosine hydroxylase expression in fawn-hooded rats (Femenia, *et al*., 2010). If a reduction in expression occurred in the present study, downstream adrenaline and NA synthesis would be hindered. Thus, this may possibly explain the reduction in catecholamine-induced arrhythmias of phase Ib recorded in rats treated with AM251 ($1mg\ kg^{-1}$).

Alternatively, activation of a non-classical cannabinoid receptor by AM251 may be responsible for the observed cardioprotection. Indeed, the studies described in Chapter 4 show clearly that at the dose used in this study AM251 is activating GPR55. AM251 is acknowledged to trigger the extracellular signal-regulated kinase, ERK, and the phosphatidylinositide-3-OH kinase (PI3K) through GPR55; these kinases are key players in the initiation of the anti-apoptotic, pro-survival kinase cascade known as the reperfusion injury salvage kinase (RISK) pathway (Cross *et al*., 2000). Activation of ERK and

PI3K in the setting of MI and reperfusion is acceded to mediate cell protection and survival (Shimiza *et al*., 1998; Yue *et al*., 2000). The means by which this is thought to be mediated include the prevention of cytoplasmic Ca^{2+} overload by promotion of SR-mediated re-uptake, inhibition of conformational changes in pro-apoptotic kinases thereby preventing apoptosis (Marzo *et al*., 1998; Yamaguchi *et al*., 2001; Tsuruta *et al*., 2002) and inhibition of the opening of the mPTP (Marzo *et al*., 1998). While it is plausible that AM251 mediates its infarct sparing effect by activation of the RISK pathway, this pathway is not linked to arrhythmia generation. However, since there is a paucity of literature between any link between GPR55 and electrophysiological changes in the heart, an action at this receptor cannot be ruled out. That being said, a recent study from Yu *et al*, (2013) showed that GPR55 activation by its endogenous ligand LPI induces mobilisation of $Ca^{2+}{}_{i.}$ If AM251 were acting as an agonist at GPR55, then these data suggest that there would be an increase in $[Ca^{2+}]_i$, which would be pro-arrhythmic, rather than anti-arrhythmic.

CBD and AM251 alone acted with the same effect on arrhythmias, albeit to different extents. They also acted in an analogous manner on MABP (as described in Chapter 4). Nonetheless, this would suggest that the effects of CBD and AM251 on MABP and VPBs are mediated by the same receptor.

No significant effects of drug treatment, either on HR prior to occlusion or during the course of ischaemia were observed and as such the protective effects of drug treatments are not likely to be due to changes in HR. Although, the protective effect of HR on ischaemia-induced arrhythmias is a contentious issue in itself high HR determined incidence of VPBs and low HRs being protected (Bolli, Fisher and Entman, 1986; Bernier, Curtis and Hearse, 1989).

5.5.3 Effect of co-administration of CBD and AM251 on ischaemia-induced ventricular arrhythmias

In this study, it was observed that the individual anti-arrhythmic capacity of CBD and AM251, were either enhanced or maintained dependent upon the order in which the compounds were administered. These data may suggest that a more complex relationship than just that of inverse agonist/antagonist at the CB_1 receptor may be occurring and encourages consideration of alternative sites of action. Moreover, the disparity between the profiles of the anti-arrhythmic effects of these agents, depending upon the order of administration, further implies the involvement of other receptors than simply the CB_1 receptor. It has previously been recognised that in a co-administration study involving a CB_1 receptor antagonist and a CB_2 receptor agonist, that each alone reduced infarct volume and that when co-administered this effect was potentiated. These authors hypothesised that there may be a degree of

synergism occurring between the two receptors (Zhang *et al*., 2008). Furthermore, the likelihood of a synergist relationship occurring between CBD and AM251 is not limited to ischaemia and reperfusion (I/R), as a previous study in our laboratory has identified possible synergism occurring in the setting of platelet aggregation (unpublished data). In particular, the modest *ex vivo* anti-aggregatory capacity of CBD was found to be significantly enhanced in the presence of AM251. Since a degree of crosstalk between the CB_1 receptor and related sites has already been established it could infer the possibility that there may be crosstalk with other related sites.

The putative cannabinoid receptor GPR55, a pharmacological target for both CBD and AM251, as an antagonist and agonist, respectively (Pacher and Hasko, 2008; Lim *et al*., 2009), may be one such target. GPR55 receptors were positively identified on endothelial cells, cardiomyocytes and inflammatory cells in both sham and ischaemic heart tissue. This confirms earlier reports of the presence of low level GPR55 receptor expression in the heart expression of this receptor predominates on the plasma membrane and membranes endo-lysosomes in ventricular cardiomyocytes and distinct signalling pathways are activated dependent upon receptor location (Yu *et al*., 2013). Our data showing GPR55 receptor staining in the heart supports this and may suggest that if AM251 and/or CBD are acting at this receptor to modulate arrhythmia occurrence, it seems likely that they are doing so directly at the level of the cardiomyocyte. In support of this, both the CB_1 receptor and GPR55 are found to interact *in vitro*. The link between CB_1 and GPR55 is determined by integrins, as described in more detail in Chapter 5. If conditions of clustered integrins persist, administration of the CB_1 and GPR55 agonist, AEA, induces CB_1-dependent activation of Syk which prevents AEA activation of GPR55-induced Ca^{2+} mobilisation from the endoplasmic reticulum (ER). If however, activation of the CB_1 receptor is prevented by the CB_1 receptor antagonist, AM251 (10 μM), integrin clustering is disengaged and there is a relief of CB_1-mediated inhibition of GPR55. This relief can unmask an AEA-induced increase in $[Ca^{2+}]_i$ by mobilisation from the ER subsequent to GPR55 activation. (Waldeck-Weiermair *et al*., 2008). The question is whether the intracellular accumulation of Ca^{2+}, which occurs during I/R, is sufficient to induce gross changes in the extracellular Ca^{2+} concentration, adequate to induce integrin clustering. This is doubtful and prevention of intracellular Ca^{2+} accumulation via an inhibition of GPR55's ability to mobilise Ca^{2+} from the ER, would irrefutably worsen the Ca^{2+} overload typical of I/R.

The notion that the cardioprotective effects of CBD and AM251 may be mediated by distinct receptors is further substantiated by data in this study which shows that CBD alone appears to suppress phase Ib re-entry-induced arrhythmias, while AM251 alone appears to suppress phase Ia catecholamine flux-induced arrhythmias. In the co-administration studies, AM251 followed by CBD showed an additive effect in terms of reducing the arrhythmia incidence during the ischaemic period, however the same was not observed in the CBD then AM251 treatment group. The observed reduction in reentry-

induced arrhythmias with CBD may be in part due to a direct electrophysiological effect, as described in detail earlier in this discussion. With regard to the AM251-mediated reduction in catecholamine-induced arrhythmias (again covered earlier in this discussion) this may have been induced by changes in the availability of NA. Again, the inconsistency in the arrhythmic phases affected by the order of administration, indicates that the order of receptor occupancy and the sites of occupancy are critical. The anti-arrhythmic effects of each alone are summative when AM251 is given before CBD, but not in the reverse order. We postulate that since both compounds act at CB_1 and GPR55, is it sensible to assume that the observed effects must in some way involve these receptors. The detailed mechanism proposed is outlined in Figure 5.21 and Figure 5.22.

Of course, the observed disparity of anti-arrhythmic capacity of co-administration of CBD and AM251 depending on the order of administration could simply be down to preferential activation of their respective pharmacological targets. To summarise their respective pharmacologies; CBD acts as an inverse agonist at CB_1 and at GPR55 as an antagonist, whereas AM251 acts as an antagonist at CB_1 and at GPR55 as an agonist. CBD is a weak inverse agonist of the CB_1 receptor (IC_{50} 3.35µM; Thomas *et al*., 2007) compared with the relatively more potent antagonist AM251 (IC_{50} 8nM), thus when AM251 is administered prior to CBD, blockade of the CB_1 receptor would likely occur. As illustrated in Figure 5.21, CBD would then be left to block GPR55, possibly preventing any significant intracellular Ca^{2+} mobilisation, achieved through occupancy by an endogenous ligand at this receptor (Lauckner *et al*., 2008). In addition, blockade of the CB_1 receptor by AM251 may facilitate the endogenous cardioprotective cannabinoids to mediate protection by preferential activation of the CB_2 receptor (Zhang *et al*., 2008; Lim *et al*., 2009). Therefore, the full extent of CB_2 receptor involvement in the mediation of the cardioprotective effects of CBD and AM251 requires further investigation.

Co-administration in the reverse order (CBD followed by AM251) did not produce the same additive effect in terms of the cardioprotection afforded when AM251 was administered before CBD. This may in part be due to pre-treatment with CBD interacting with and blocking tonic activity at the CB_1 receptor (and thus preventing AM251 from gaining access to the receptor site). Activation of CB_1 has been shown to be proarrhythmic in a model of global ischaemia in the isolated rat heart (Andrag and Curtis, 2013). Figure 5.21 and Figure 5.22 illustrates a proposed hypothesis to explain the current findings. We hypothesise that CBD may be acting principally at the CB_1 receptor if it is given prior to AM251 administration and, although the beneficial or indeed detrimental role of the CB_1 receptor in I/R remain disputed, the blockade of tonic activity by CBD might be responsible for the conferred anti-arrhythmic effect. Consequently, when AM251 is then administered the remaining site of action could be GPR55, resulting in the potentiation of ischaemia-induced arrhythmias quantified over the 30 minute ischaemic period. Indeed GPR55 activation has been identified as a potential mediator of cell injury (Kapur et al., 2009; Sharir and Abood, 2010).

Alone, CBD and AM251 bolus administration produced a small reduction in VF incidence. The effects of CBD on VF were markedly enhanced by AM251 (AM251 then CBD), however co-administration in the reverse order (CBD then AM251) completely reversed the small reduction in VF mediated by AM251. These data would suggest the involvement of more than just the CB_1 receptor in the mediation of reduction in VF incidence.

AM251 then CBD

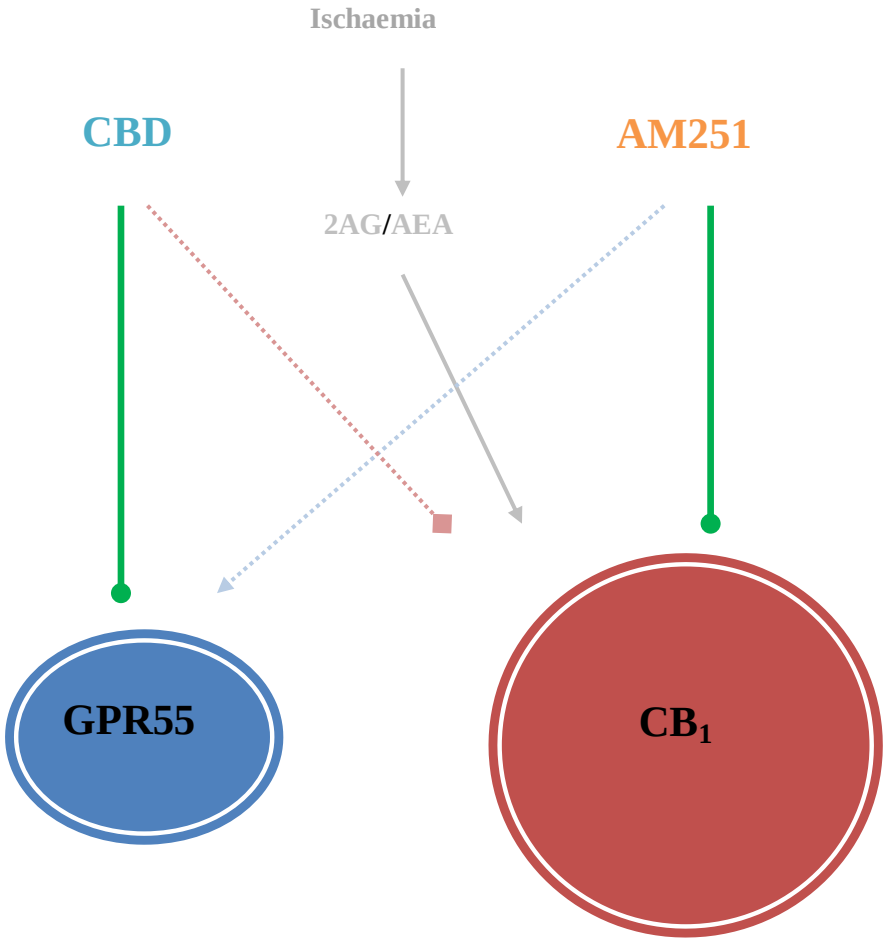

Figure 5.21 Proposed pharmacological activation of the CB_1 receptor and GPR55 depending on the order of administration of CBD and AM251. It is proposed that when AM251 administration preceeds that of CBD, there is pharmacological blockade (antagonism – green arrow) of the CB_1 receptor by AM251. This would allow 2AG and AEA (whose production is upregulated as a consequence of ischaemia) to activate CB_2 receptor exclusively. The second effect may involve CBD acting at GPR55 as its site of action at CB_1 is no longer available as a result of occupation by AM251. CBD would antagonise (green arrow) GPR55 potentially preventing intracellular Ca^{2+} mobilisation induced by an endogenous ligand such as LPI. Dashed arrows indicate the additional pharmacological actions of CBD, AM251 and the endocannabinoids, those actions being prevented by the proposed activity indicated by the bold arrows (blue arrow – agonism; red arrow – inverse agonism, grey arrow – agonism).

CBD then AM251

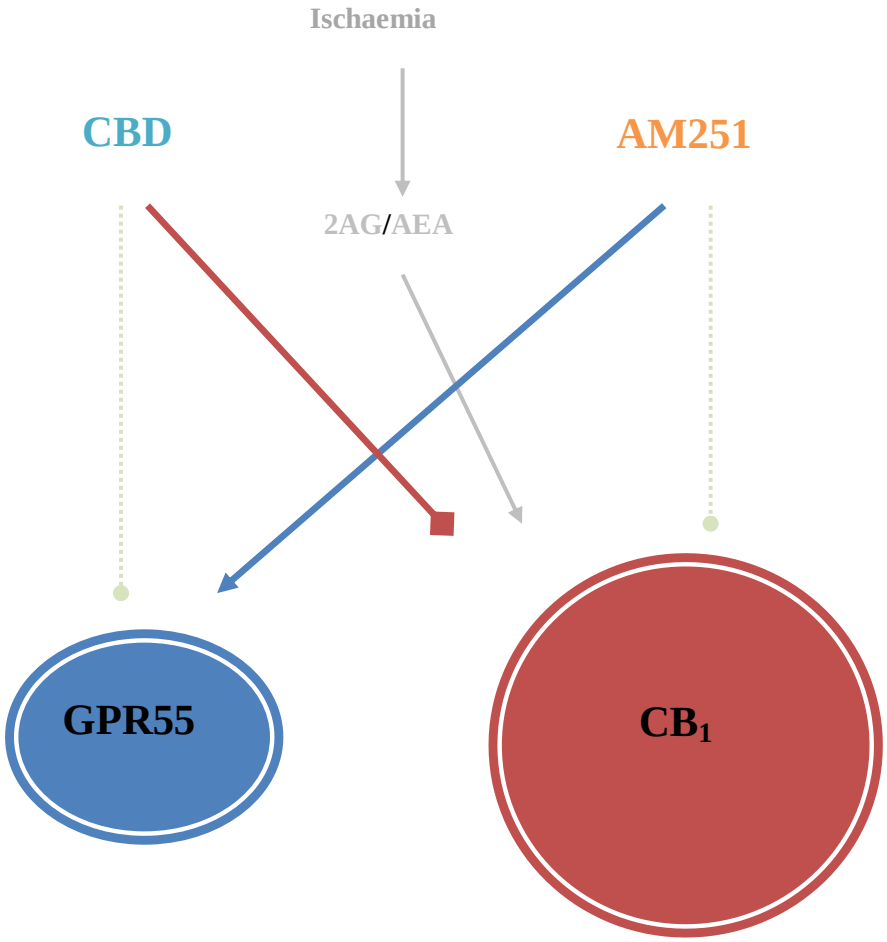

Figure 5.22 Proposed pharmacological activation of the CB_1 receptor and GPR55 depending on the order of administration of CBD and AM251. We propose that when AM251 is given after bolus CBD administration, CBD acts principally at CB_1 receptors as an inverse agonist (red arrow), thus preventing constitutive receptor activity. CB_1 receptor activation in MI has been demonstrated to be proarrhythmic (Andrag and Curtis, 2013). What is more, AM251 when administered could act as an agonist at GPR55 (blue arrow). GPR55 has been implicated in the development of arrhythmias in myocardial ischaemia. Dashed arrows indicate the additional pharmacological actions of CBD, AM251 and the endocannabinoids, those actions being prevented by the proposed activity indicated by the bold arrows (green arrow – antagonism; grey arrow – agonism).

5.5.4 Summary and conclusions

This study confirmed previous observations which showed that CBD is anti-arrhythmic and is the first to demonstrate that AM251 is cardioprotective in an anaesthetised rat model of MI. Moreover, co-administration of CBD and AM251, in either order was identified as anti-arrhythmic. Interestingly, the order of administration of CBD and AM251 appears to affect the degree of potentiation of anti-arrhythmicity of either compound alone. None of the drug treatment regimes had any significant effect on recovery of MABP following ligation of the coronary artery. However, attempts to identify the receptors responsible for mediating the anti-arrhythmic effects of either drug were hampered by the complex pharmacology of the two compounds, which in both cases involved CB_1 and GPR55 and the likelihood of 'cross-talk' between the two receptors. As a result the next series of studies sought to explore the role of $[Ca^{2+}]_i$ in the action of both CBD and AM251.

6. Development of a method to measure changes in $[Ca^{2+}]_i$ in isolated rat ventricular myocytes

6.1 Introduction

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays a fundamental role in the propagation of the spontaneously generated action potentials of the SA node, while the inward movement of Ca^{2+} in atrial and ventricular myocytes creates the prolonged depolarisation typical of these cells, known as the plateau phase (Irisaw, Brown and Giles, 1993; Levick, 2003; Pollock and Richards, 2004; Figure 1.2). The electrical excitation of cardiac myocytes initiates contraction in a process termed excitation-contraction coupling (Bers, 2002). The synchronous contraction of atrial and ventricular syncytiums ensures the efficient pumping of the heart and propulsion of blood to the lungs and systemic circulation (Levick, 2003; Pollock and Richards, 2004). The reliance of the heart on Ca^{2+} was first identified in 1883 (Ringer), and since that discovery it has been shown that without extracellular Ca^{2+}, Ca^{2+}-induced rises in intracellular Ca^{2+} cannot occur, leading to prevention of myocardial contraction (Levick, 2003).

Perturbation of physiological concentrations of Ca^{2+} rapidly results in deterioration of cardiac cell physiology. Increased Ca^{2+} in cardiac myocytes predisposes the heart to electrical instability and, consequently the likelihood of triggered automaticity, reentry and electrical inhomogeneity leading to subsequent ventricular fibrillation and tachycardia (Lubbe, Podzuweit and Opie, 1992; Ugdyzhekova *et al*., 2001). $[Ca^{2+}]_i$ overload subsequent to a period of ischaemia can also result in reoxygenation-induced contracture in rat perfused hearts (Steenbergen *et al*., 1990). Hypercontracture is a perpetual, ischaemia-induced, stiffening of the myocardium which, if persistent enough, inhibits the recovery of contractile function of the myocardium (Ladilov *et al*., 2003). Thus, given the fundamental role $[Ca^{2+}]_i$ plays in the electrophysiology and mechanical function of the heart, it is important to consider the role it may play in the mediation of the anti-arrhythmic effects of CBD and AM251 that were described in Chapter 5.

6.1.1 Myocardial ischaemia and Ca^{2+}

As detailed in Chapter 1, the electrophysiological changes induced by an episode of acute MI begin with the extracellular accumulation of K^+ within 10 seconds of cessation of coronary flow (Hill and Gettes, 1980; Hirche *et al*., 1980; Nayler, 1981; Fozzard and Makielski, 1985; Janse, 2000). This begins a cascade of ionic disturbances, with the rise in $[Ca^{2+}]_i$ occurring subsequent to the extracellular $[K^+]_e$ rise and intracellular $[Na^+]_i$ accumulation and with comparative delay to K^+ and Na^+ (Allen and Orchard, 1983). Concomitant to these ionic changes, there is a depolarisation of cells within the ischaemic zone, in a process thought consequent to alterations in $[K^+]_e$ (Kleber, 1983; Coronel *et al*., 1988; Janse and Wit, 1989).

In the early stages of acute MI the perturbation in $[Ca^{2+}]_i$ is caused initially by displacement of Ca^{2+} from its intracellular cytoplasmic buffer sites by accumulating H^+ (Gambassi *et al*., 1993; Isenberg *et al*., 1993; Carmeliet, 1999). Reversal of the Na^+-Ca^{2+} exchanger, induced in compensation for the concurrent rise in $[Na^+]_i$, then further increases $[Ca^{2+}]_i$, as demonstrated in isolated rat heart and isolated ferret papillary muscle (Figure 6.1; Smith and Allen, 1988; Tani and Neely, 1989; Haigney *et al*., 1994; Chen and Li, 2012; Ottolia *et al*., 2013). Finally, the inadequate SR re-uptake of Ca^{2+}, following slowing of the ATP dependent Ca^{2+} pump due to reduction of the maximal rate (V_{max}) of Ca^{2+} movement, increases $[Ca^{2+}]_i$ further still (Kaplan *et al*., 1992; Carmeliet, 1999; Ottolia *et al*., 2013).

Thandroyen et al. (1992) used an isolated, cultured rat ventricular myocyte model to determine the sequence of ionic alterations which transpire as a consequence of hypoxia, and how these relate to the initiation and development of cellular injury. In this model, metabolic inhibition by hypoxia resulted in a loss of intracellular K^+ and magnesium ions (Mg^{2+}) with a concomitant gain of intracellular Na^+ and Ca^{2+} (Buja *et al*., 1985; Morris *et al*., 1989; Jones *et al*., 1989; Buja *et al*., 1990; Thandroyen *et al*., 1992). The changes in $[Ca^{2+}]_i$ induced by hypoxia in isolated rat ventricular myocytes are dependent on simultaneous extracellular acidosis (Pravdic, Vladic and Bosnjak, 2009). These biochemical changes are similar to those observed in both *in vitro* and *in vivo* models of hypoxia (Buja *et al.*, 1985; Morris *et al.*, 1989; Jones *et al.*, 1989; Buja *et al.*, 1990; Thandroyen *et al.*, 1992). Thus it is clear that changes in $[Ca^{2+}]_i$, by the induction of MI, can have a significant and profound effect on the electrophysiology of the myocardium, which ultimately provides an environment ideal for the generation of VPBs.

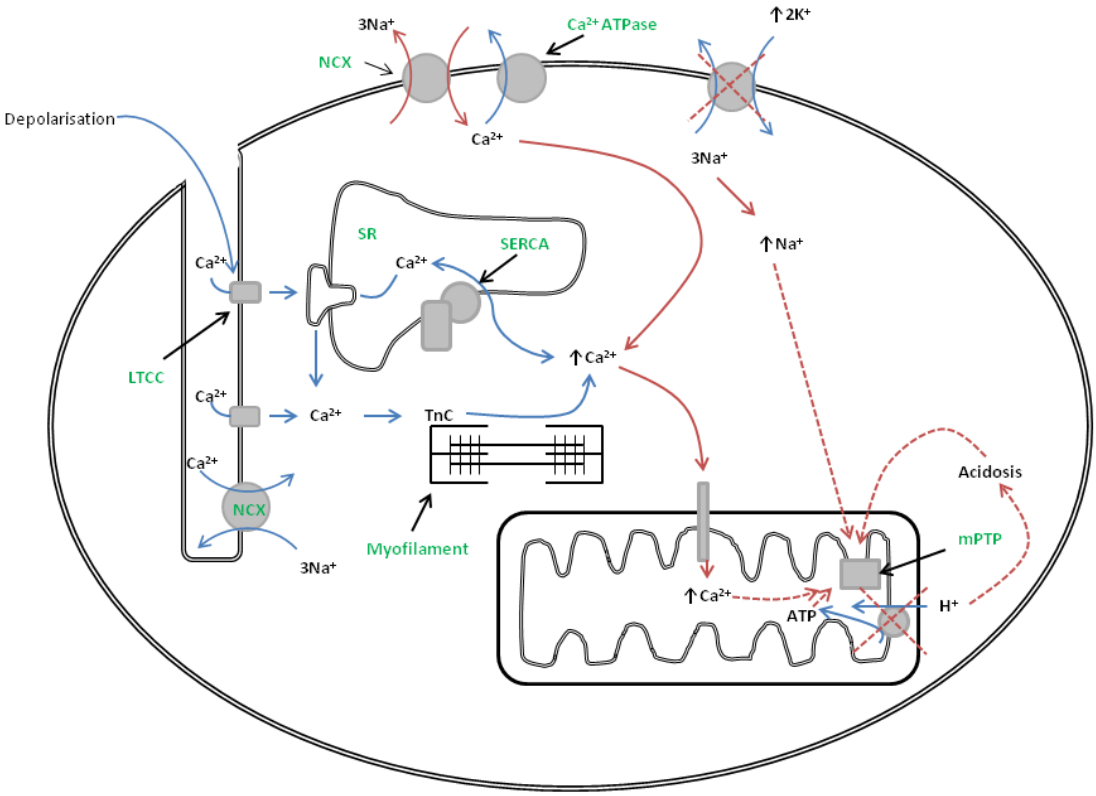

Figure 6.1 Schematic of Ca^{2+} signalling in the cardiac myocyte (blue arrows) and changes in this signalling induced by MI (red arrows). Depolarisation of the area around the t-tubule begins the process of contraction of the cardiac myocyte through stimulation of Ca^{2+} entry by way of the voltage-dependent LTCC. Ca^{2+} entry triggers further rises in cytosolic Ca^{2+} by ryanodine receptor-mediated release from the SR. The intracellular Ca^{2+} binds to the myofilaments initiating contraction. In the setting of MI there is a reversal of Na^{+}-Ca^{2+} exchanger (NCX) and inadequate SR reuptake of Ca^{2+} by the sarcoplasmic reticulum calcium transport ATPase (SERCA), which leads to cytosolic and mitochondrial Ca^{2+} overload. These perturbations coupled with the loss of Na^{+}-K^{+} ATPase and acidosis, leads to the increased likelihood of mPTP opening in the IMM. Adapted from Crompton, 1999; Bers, 2000; Suleiman, Halestrap and Griffiths, 2001.

6.1.2 Effect of accumulating $[Ca^{2+}]_i$ following acute myocardial ischaemia

Changes in ion flux induced by acute ischaemia change the basic electrophysiological properties of the cardiac cell (Janse and Wit, 1989). This results in a reduction in conduction velocity, variability in refractoriness and provides a suitable milieu for the genesis of arrhythmias (Czarnecka, Lewartowski and Prokopczk, 1973; Downer, Janse and Durrer, 1977; Kleber *et al*., 1978; Janse and Wit, 1989). Indeed, accumulation of $[Ca^{2+}]_i$ in isolated cardiomyocytes (to micromolar concentrations), has been found to precede the commencement of spontaneous fibrillation in neonatal rat myocytes. This intracellular accumulation of $[Ca^{2+}]_i$, as a consequence of the progressive extracellular $[Ca^{2+}]$ elevation, was used to identify the role $[Ca^{2+}]_i$ played in the development of premature beats (Thandroyen *et al*., 1991). Increased $[Ca^{2+}]_i$ is typically found after a period of MI (Steenbergen *et al*., 1990; Carmeliet, 1999). In fact, elevated $[Ca^{2+}]_i$ has been identified as a mediator in the induction of triggered automaticity, reentry and electrical inhomogeneity (Clusin, Buchbinder and Harrison, 1983; Opie *et al*., 1988; Thandroyen *et al*., 1991), which are critical precursors to the occurrence of VF and VT (Lubbe, Podzuweit and Opie, 1992).

In addition to the electrophysiological abnormalities that increased $[Ca^{2+}]_i$ can produce, additional pathogenic alterations may occur which can limit the recovery of the post-ischaemic myocardium. The prevention of adequate ATP synthesis, coupled with the ionic imbalances that occur during MI, can impair the regulatory processes that maintain baseline $[Ca^{2+}]_i$ within the 10^{-7}M range in the cardiac myocyte (Steenbergen *et al*., 1990). As detailed above, MI is associated with increases in $[Ca^{2+}]_i$ and it is this accumulation of $[Ca^{2+}]_i$ that can induce Ca^{2+} overload-induced contracture upon reperfusion (Ladilov *et al*., 2003; Piper, Meuter and Schafer, 2003). The high $[Ca^{2+}]_i$ coupled with re-activation of ATP synthesis upon reperfusion leads to uncontrolled initiation of contraction in cardiac myofibrils ultimately leading to the shortening and stiffening of the myocardium which is termed contracture (Steenbergen *et al*., 1990; Ladilov *et al*., 2003; Piper, Meuter and Schafer, 2003). The development of contracture in the post-ischaemic myocardium, if persistent, can lead to cytoskeletal defects that cause structural instability in cardiac myocytes (Piper, Meuter and Schafer, 2003).

High $[Ca^{2+}]_i$ following MI can induce opening of the mPTP which causes apoptotic and necrotic cell death upon reperfusion of the ischaemic myocardium (Hausenloy, Duchen and Yellon, 2003). The uptake of Ca^{2+} by mitochondria during a period of ischaemia is the crucial factor that will ultimately predict the death or survival of the cardiomyocyte, since this is the driving force in changing the probability of the opening of the mPTP (Crompton and Costi, 1988; Hausenloy, Duchen and Yellon, 2003).

6.1.3 Cannabinoids and $[Ca^{2+}]_i$

The determination of survival of cardiac myocytes following an episode of acute MI can thus be, at least in part, attributed to the maintenance of homeostasis of $[Ca^{2+}]_i$ (Janse and Wit, 1989; Carmeliet, 1999; Hausenloy, Duchen and Yellon, 2003). What is more, calcium-mediated perturbations in normal electrophysiology, for example slowing of the action potential, early and delayed after depolarisations and reentry, have been implicated in the development of arrhythmias (Thandroyen *et al*., 1991; Kranias and Bers, 2007). Reduction of $[Ca^{2+}]_i$ through the use of Ca^{2+} channel blockers have been shown to reduce arrhythmia incidence in an isolated multicellular cardiomyocyte model (Thandroyen *et al*., 1991). Cannabinoids (synthetic, endogenous and plant-derived alike) have been shown to act on both Ca^{2+}-dependent processes and on Ca^{2+} homeostasis on post-synaptic cells of the nervous system, at the TRPV1 receptor and in the heart (Brenowitz and Regehr, 2003; van der Stelt *et al*., 2005; Currie *et al*., 2008; Drysdale *et al*., 2009). Ca^{2+} signalling implicated in the generation of arrhythmias in rats has been linked to IP_3 receptor mediated signalling (Mackenzie *et al*., 2002) and furthermore, endothelin-induced arrhythmogenic Ca^{2+} mobilisation in mice can be ameliorated in IP_3 receptor knock-out mice (Li *et al*., 2005). Interestingly, IP_3 receptor-mediated Ca^{2+} mobilisation can also be modified by AEA and AM251 (Currie *et al*., 2008), suggesting a role for cannabinoids in the modulation of Ca^{2+} signalling. Indeed, CBD has been shown to alter $[Ca^{2+}]_i$ in numerous cell types including, but not limited to, hippocampal neurons, leukocytes and mast cells (Kaplan *et* al., 2003; Drysdale *et al*., 2006; Giudice *et al*., 2007), although to date there is no data on its effect on Ca^{2+} in cardiomyocytes. CBD is known to effect $[Ca^{2+}]_i$ changes by modulating NMDA-elicited Ca^{2+} signals and through inhibition of voltage-gated calcium channels (Mackie and Hille, 1992; Twitchell, Brown and Mackie, 1997; Netzeband *et al*., 1999; Drysdale *et al*., 2006). In neurons and glial cells, CBD causes an increase in $[Ca^{2+}]_i$ that is sustained even after washout of CBD, and is independent of an action at CB_1 receptors (Drysdale *et al*., 2006) and is believed to be due to release of Ca^{2+} from intracellular stores. In support of the latter, Ryan et al. (2009) demonstrated that application of CBD to primary rodent hippocampal cells kept under conditions of high excitability, reduced $[Ca^{2+}]_i$ and prevented $[Ca^{2+}]_i$ oscillations, leading them to propose that the intracellular site of action of CBD was principally the mitochondrial sodium-calcium exchanger.

Additionally, AM251 has been shown to prevent dysfunction of mitochondria in a model of hepatic lipogenesis (Chen, Ho and Lee, 2013) and CBD can modulate $[Ca^{2+}]_i$ depending on the excitability of the cell in a manner in part aided by mitochondria (Ryan *et al*., 2009).

Thus, in light of the anti-arrhythmic effects observed with CBD and AM251 in this present study, this study explored their potential to modulate Ca^{2+} and mitochondrial membrane potential as a possible mechanism underlying their anti-arrhythmic effects.

6.1.4 Use of an *ex vivo* model of simulated ischaemia in freshly isolated rat cardiomyocytes

In light of the findings from the *in vivo* studies (described in Chapter 5) which identified the anti-arrhythmic capacity of CBD and AM251 it was postulated that a potential mechanism of their action was to stabilise $[Ca^{2+}]_i$ following induction of acute MI. Furthermore, in light of the pharmacological targets of both CBD and AM251 being similar, namely CB_1 receptors and GPR55, it was pertinent to attempt to investigate the involvement of these receptors in any observed changes in $[Ca^{2+}]_i$ during normoxia and simulated ischaemia. Thus the aims of this study were to:

- To optimise a method for the isolation of ventricular cardiomyocytes from the rat heart,
- To develop a method to detect changes in cardiomyocyte $[Ca^{2+}]_i$ using fluorescent microscopy,
- To establish an *ex vivo* model of simulated ischaemia (in an effort to mimic the effects of oxygen deprivation induced by acute, regional MI *in vivo*) to determine the effects of this pathophysiological state on $[Ca^{2+}]_i$ in isolated cardiomyocytes,
- A further aim was to identify whether CBD and/or AM251 affect basal $[Ca^{2+}]_i$ or any changes in $[Ca^{2+}]_i$ mediated by simulated ischaemia and whether these changes are mediated by CB_1 or GPR55 receptors using a similar pharmacological approach to that used for the arrhythmia studies.

6.2 Methods

6.2.1 Preparation of cell extracts from adult rat ventricles and detection of intracellular Ca^{2+} in isolated ventricular myocytes

Male Sprague Dawley rats (n=29; before exclusion criteria were applied; final n numbers can be found in the appropriate results section) were euthanised by CO_2 asphyxiation as described in section 2.4.1.

6.2.2 Enzyme digestion and isolation of adult rat ventricular cardiomyocytes

The method of cell isolation from adult rat ventricles is described in Chapter 2. Isolation of cells was achieved by a modification of the original protocol described by Bates and Gurney et al., (1999), specifically:

1. To improve cell yield and cell viability during the isolation process, a number of modifications were made to the technique; firstly, perfusion times for each of the solutions were extended to allow adequate time for perfusate to percolate through the entirety of the tissue mass. Perfusion extensions were thus, 6 minutes with Ca^{2+}-free solution, 3 minutes with Ca^{2+}-free solution containing proteinase and 11 minutes with Ca^{2+}-free solution containing collagenase and hyaluronidase.
2. Upon conclusion of ventricular mincing, the tissue was transferred to a microcentrifuge tube where it was bathed in Krebs-Henseleit solution and gently titurated with a glass-fired pipette prior to placing in an incubator at 37°C until required for fluorescence staining. The original protocol from which these modifications were made is detailed in appendix II.

Viable cells were those which showed typical morphology of a cardiomyocyte when observed under a light microscope, namely an elongated, rod-shaped cell, with a smooth outer edge. Cardiomyocytes which have been over digested during enzymatic dispersion will typically be more spherical in shape. The identification of striations also helped to confirm the successful isolation of the correct cell type (Figure 6.3).

6.2.3 Identification of the effect of CBD and AM251 on the mitochondrial membrane potential

Having obtained viable cardiomyocytes, a series of experiments was performed that sought to identify the effect of CBD and AM251 on mitochondria membrane potential using redox sensor red CC-1 (CC-1), a redox sensitive fluorescent dye which selectivity accumulates in mitochondria (and lysosomes) following oxidisation in the cytosol of the cell (Shukla *et al*., 2003). The hypothesis for this series of experiments was that CBD or AM251 may be preventing or delaying depolarisation of the mitochondria due to mPTP opening, which typically occurs following ischaemia (Siddall *et al*., 2013). Thus, CC-1 was used as a detector of mitochondrial membrane potential and FCCP was used to uncouple the mitochondria in order to identify if CBD or AM251 could reduce membrane depolarisation. The initial experiments aimed to identify the optimal concentrations of CC-1 and the mitochondrial uncoupling agent, FCCP, the latter of which was used to indicate the effect of mitochondrial uncoupling, on fluorescence intensity. The concentrations of CC-1 used were 1, 3 or 5µM and the concentration range of FCCP tested was 10 and 1µM and 100nM. Cells were isolated as described in section 6.2.2. 300µl of cell suspension was pipetted into each well of the 96 well plate (Millipore, Massachusetts, USA), cells were allowed 1, 3 or 5 hours to become adherent in an incubator at 37°C. Plates were laminin coated by incubating each well with 50µl with laminin overnight. FCCP was incubated (37°C) for 10 minutes prior to CC-1 addition. CC-1 was incubated for 10 minutes at 37°C. The bath solution was pipetted off from each well before cells were washed once in Krebs solution; each well was then refilled with 300µl of Krebs solution. Fluorescence intensity was read using a Bio-tek FL600 microplate flourescence reader (MTX Lab Systems Inc, Virginia, USA) the data for which was relayed to a desktop computer equipped with CS4 software. Plates were read from below and sensitivity was set to 25, excitation wavelength was set to 560/20 and emission wavelength to 590/20.

6.2.4 The effect of CBD and AM251 on $[Ca^{2+}]_i$ in normoxia and simulated ischaemia

The next series of experiments were designed to try and assess cardiomyocyte $[Ca^{2+}]_i$ using fluorescent microscopy in both normoxia and simulated ischaemia. The experimental design was changed from that described in section 6.2.3 because of the difficulty in determining significant changes in fluorescent intensity between blank and CC-1 loaded cardiomyocytes. While an inital aim during design of the CAO experiments, was to investigate both the electrophysiological (anti-arrhythmic) and tissue sparing (infarct size) of CBD, in the interest of time, the study only investigated the former and

so, study of a tissue sparing end-point (effect on mPTP) in the isolated cardiomyocytes was no longer applicable. Therefore the experiments were designed to investigate any effect of CBD and AM251 on ischaemia-induced changes in $[Ca^{2+}]_i$, that have been identified as a trigger for multiple forms of ectopic activity (Clusin, Buchbinder and Harrison, 1983; Opie *et al*., 1988; Thandroyen *et al*., 1991; Lubbe, Podzuweit and Opie, 1992).

Figure 6.2 illustrates the experimental protocol used in this study. Healthy cells isolated from ventricular tissues were subject to investigation of the effect of a single drug treatment on $[Ca^{2+}]_i$. Experimental drug treatment groups were; (i) vehicle control (0.002% dimethyl sulfoxide and 0.25% ethanol (EtOH)), (ii) CBD (3µM), (iii) AM251 (25µM), (iv) CBD in the presence of AM251, (v) AM251 in the presence of CBD. $[Ca^{2+}]_i$ was detected by loading isolated ventricular cardiomyocytes with the acetoxymethyl (AM) ester of fluo-4 as described in section 2.4.2. Fluo-4 AM (5µM) was used as tool to determine $[Ca^{2+}]_i$ changes in isolated ventricular cardiomyocytes and thus changes in fluo-4 AM fluorescence are referred to as changes in $[Ca^{2+}]_i$. All drug treatments were added to the cell-fluo-4 AM suspension after 20 minutes of fluo-4 AM incubation and the remainder of the incubation period occurred at room temperature. 100µl of the fluorescence-loaded labelled cells were then pipetted onto a perfusion chamber mounted on the stage of a Leica DMI 4000B microscope. These cells were brought into focus at x200 magnification using phase light before being illuminated with fluorescent light (100w Hg lamp) filtered by the blue-green-red filter cube (excitation band Pass 495/15; dichromatic mirror 510 and suppression filter 530/30).

The movie setting of the Leica image capture software was then initiated to capture the pre-wash cell fluorescence. Cells were then superfused with Krebs-Henseleit solution at a rate of 1ml min^{-1}. Images of cells were recorded during this wash period. The bath solution was continually aspirated off by way of a Dymax air pump (Charles Austen Pumps Ltd, Surrey, UK). Cells were finally stimulated by switching to a Krebs-Henseleit solution (Table 6.1) containing 60mM KCl and image sequences recorded. All normoxic solutions were maintained by bubbling solutions with a 95% O_2 and 5% CO_2 gas mix.

For simulation of ischaemia, the experimental protocol was comparable to that for normoxic cells with the exception that Krebs-Henseleit solution was replaced with an ‘ischaemic’ bath solution at the wash stage of the protocol (Table 6.1) in that the solutions in these series of experiments were gassed with 95% N_2/5% CO_2.

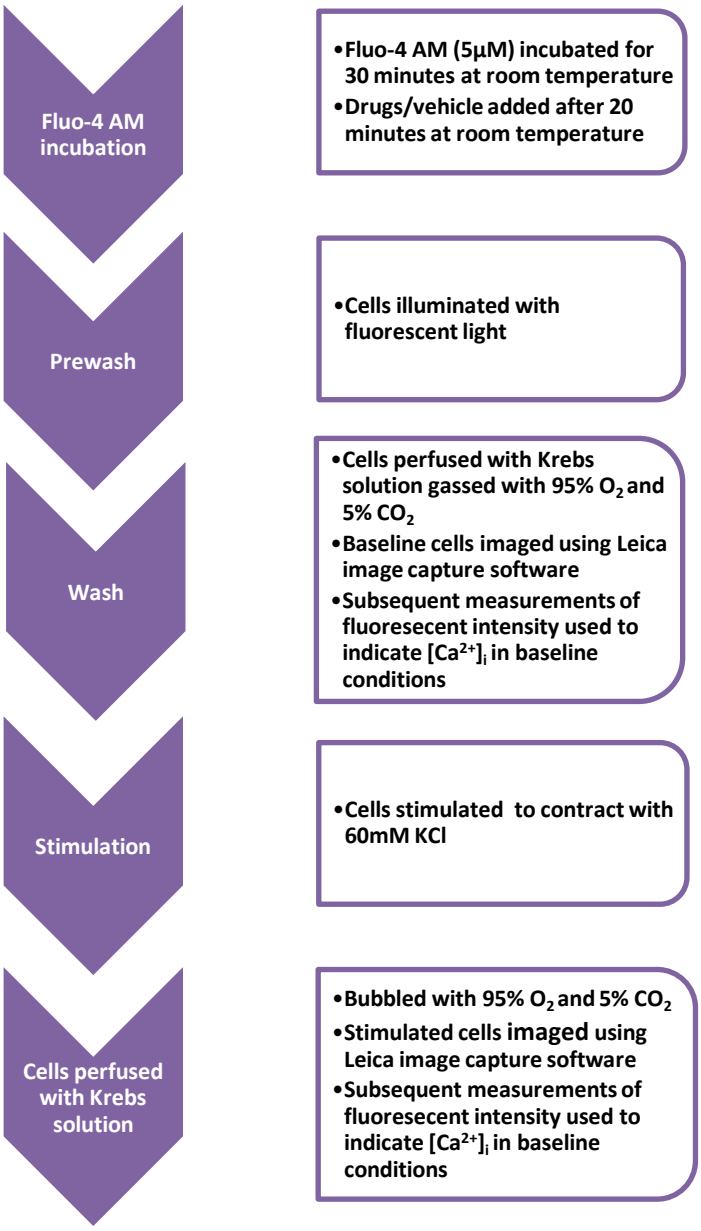

Figure 6.2 Drug treatment and experimental protocol for normoxic isolated cardiomyocytes

Krebs-Henseleit buffer solution and ischaemic bath solution

Salt	*Normoxic concentration*	*Ischaemia concentration*
NaCl	120mM	141.5mM
KCl	2.5mM	8mM
$MgCl_2$	2.5mM	1mM
NaH_2PO_4	0.5mM	1.2mM
KH_2PO_4	0.5mM	-
$NaHCO_3$	15mM	8.5mM
$CaCl_2$	1mM	1.8mM
Glucose	10mM	5.5mM
HEPES	5mM	-
Distilled water	1000ml	1000ml
pH	7.4	6.8

Table 6.1 Composition of Krebs-Henseleit solution and the ischaemic bath solution

6.2.5 Exclusion criteria

The exclusion criterion for these data was a lack of change in fluorescence to stimulation with 60mM potassium chloride (KCl).

6.2.6 Data analysis and expression

Images were sampled at a frequency of 1Hz. All images were normalised to background fluorescence. Fluo-4 AM fluorescence in cardiomyocytes was initially calculated as the grey scale value over the length of 17 pixels in each myocyte, and the same measurement was taken from a cell-free area of each image to allow for normalisation. Mean changes from baseline in response to both drug additions

and subsequent KCl stimulation were then calculated in conditions of normoxia and simulated ischaemia.

To determine any changes in baseline $[Ca^{2+}]_i$ by the drug intervention, results were expressed as fold change in florescence compared with baseline value (F/F_0), where F is the measured, unstimulated fluorescence in drug-treated cells and F_0 is the unstimulated fluorescence in separate vehicle-treated cells.

$$Fold\ change\ in\ baseline\ fluoresence\ (F/F_0) = \frac{Baseline\ fluoresence\ of\ drug\ treated\ cells}{Baseline\ fluoresence\ of\ vehicle\ control\ cells}$$

Equation 5.1 Equation for calculation of fold changes in baseline fluorescence in isolated cardiomyocytes.

For cells stimulated with 60mM KCl, results were expressed as fold changes in peak fluorescence compared with baseline value (F/F_0), where F is the fluorescence measured after drug addition and F_0 is the corresponding baseline fluorescence for that drug treatment.

$$Fold\ change\ in\ peak\ fluorescence\ (F/F_0) = \frac{Peak\ fluorescence\ of\ drug\ treated\ cells}{Baseline\ fluorescence\ of\ drug\ treated\ cells}$$

Equation 5.2 Equation for calculation of fold changes in peak fluorescence in isolated cardiomyocytes.

For the fluorescence intensity data from the CC-1 experiments, average well fluorescence intensity was measured in triplicate for each treatment and then repeated in cells from at least 3 more rat hearts.

6.2.7 Statistical analyses

Statistical analyses were performed on all data sets with a one-way ANOVA and Dunnett's post-hoc test and significance accepted as $P<0.05$. The exception to this was the analysis of the effect of ischaemia on baseline and peak fluorescence in vehicle control cells, which was analysed using a Student's t test. Group sizes before application of exclusion criteria were as follows; Vehicle (n=6); CBD (n=5); AM251 (n=5); CBD then AM251 (n=4) and AM251 then CBD (n=5). Fluorescence for each intervention was measured in more than one cell (usually 2-3). These readings were then averaged to produce a single n number for each experiment, with the final n numbers stated being representative of the total number of hearts per group.

6.3 Results

6.3.1 Identification of rat ventricular cardiomyocytes

Figure 6.3 shows an example of a viable cardiomyocytes isolated by enzymatic dispersion described in section 6.2.2. This rod-shaped cardiomyocyte has been incubated with fluo-4 AM which is dispersed uniformly through the cardiomyocyte. The dark and light striated pattern shows the parallel actin and myosin filaments.

6.3.2 Determination of optimum CC-1 and FCCP concentrations for quantification of changes in mitochondrial membrane potential

To optimise the concentration of CC-1 isolated cardiomyocytes were loaded with a concentration range of 1, 3 and 5 µM were tested. There was no significant difference in fluorescence intensity between any of the concentrations tested (35 ± 7, 35 ± 5, 30 ± 9 vs. 41 ± 9; CC-1 1, 3 and 5µM, respectively vs. blank; Figure 6.4), thus, the subsequent experiments were completed at the lowest concentration of CC-1. Addition of FCCP had no significant effect on fluorescence intensity at any concentration, compared with either blank or CC-1 (1µM)-treated cells (Figure 6.5).

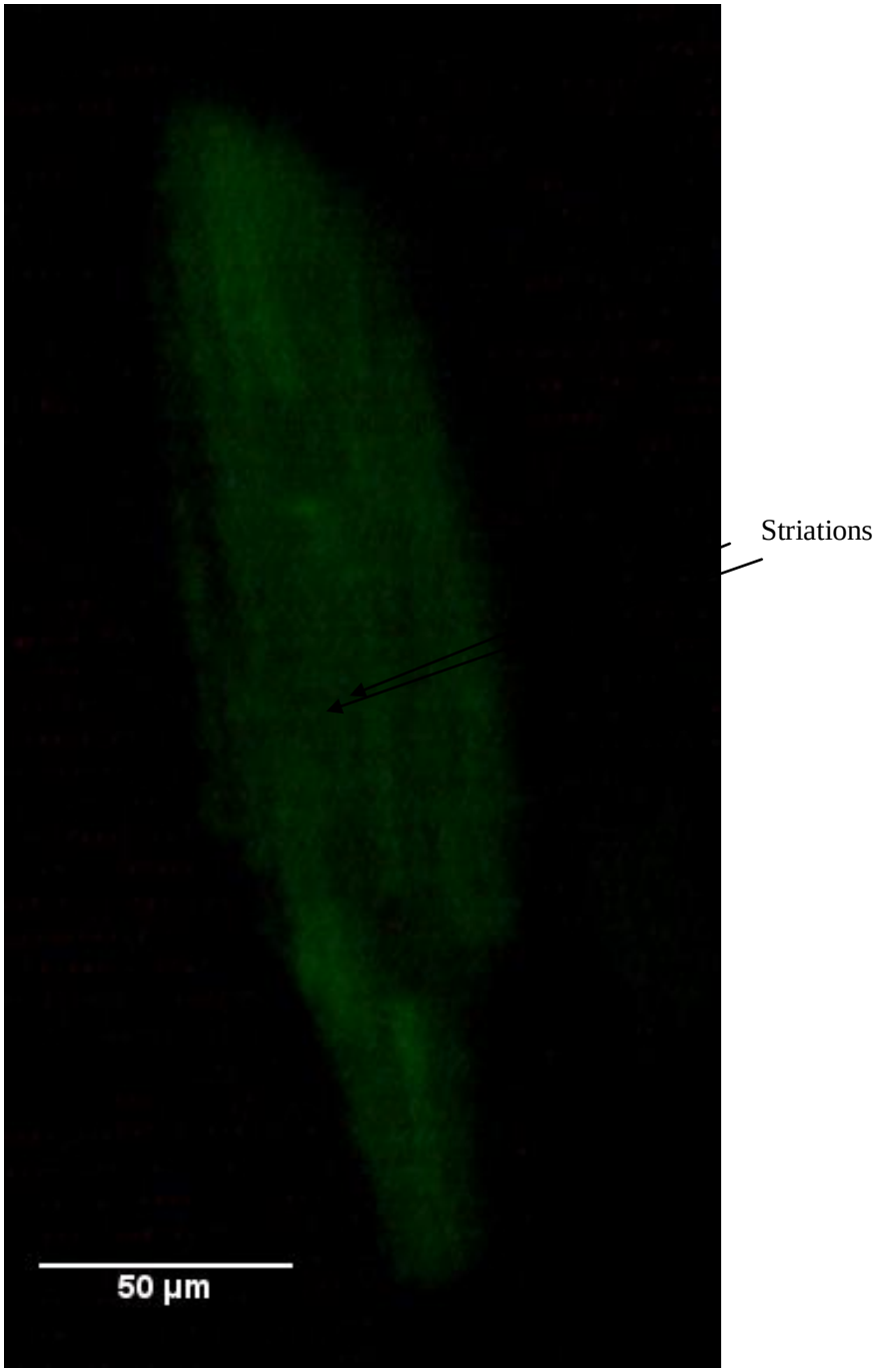

Figure 6.3 An example adult rat ventricular cardiomyocyte showing striated banding. Ventricular cardiomyocytes were isolated by enzymatic dispersion and tituration, incubated with fluo-4 AM and viewed at x200 magnification.

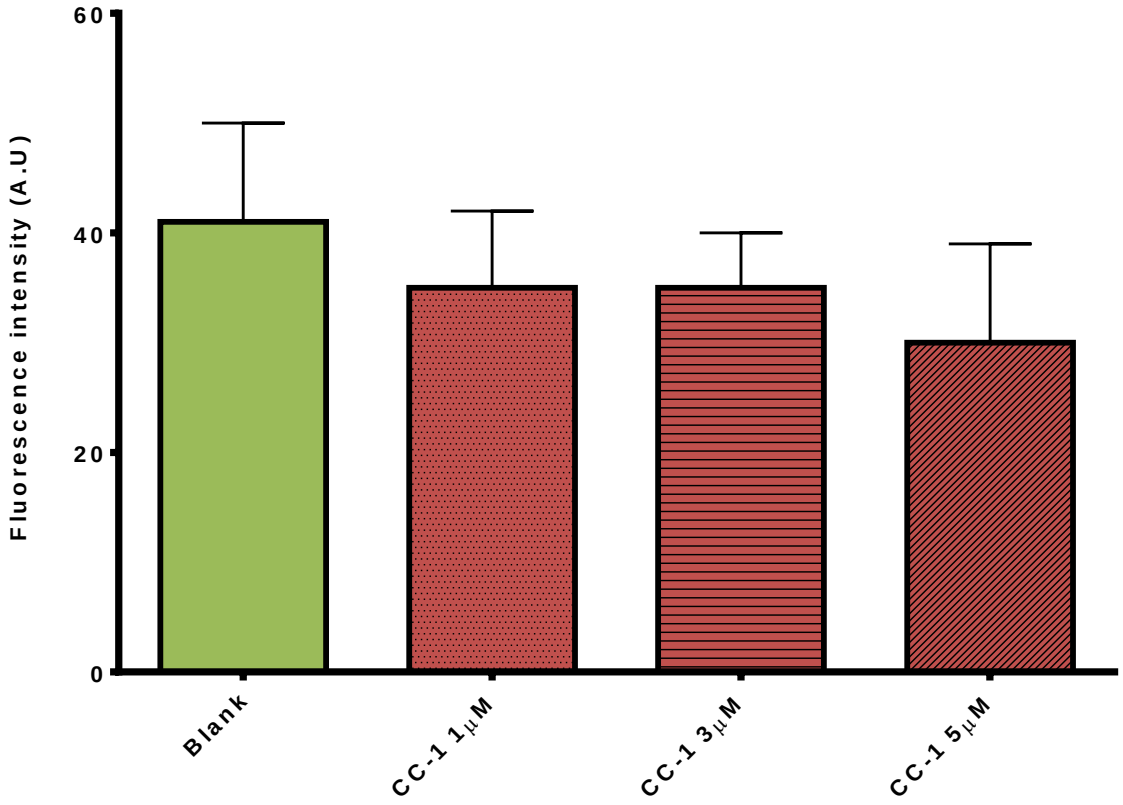

Figure 6.4 The effect of increasing concentrations of CC-1 on fluorescence intensity. Isolated ventricular cardiomyocytes were incubated with different concentrations of CC-1 to determine the optimal concentration for the quantification of fluorescence intensity. Data are expressed as mean fluorescence intensity ± SEM (arbitrary units; A.U). Samples were measured in triplicate, n=5.

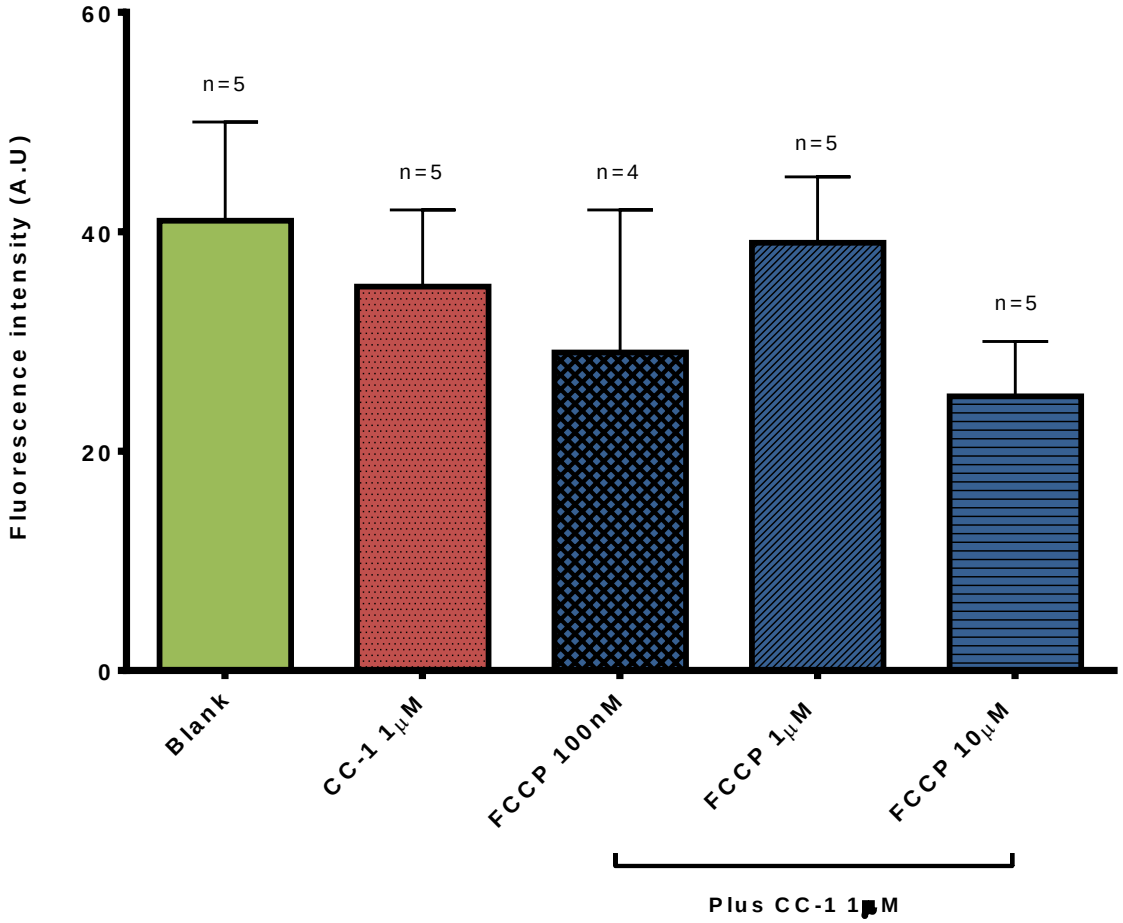

Figure 6.5 The effect of increasing concentrations of CC-1 fluorescence intensity. Isolated ventricular cardiomyocytes were incubated with CC-1 and increasing concentrations of FCCP to determine the optimal concentration of FCCP to induce significant changes in CC-1 fluorescence intensity. Data are expressed as mean fluorescence intensity ± SEM (arbitrary units; A.U). Samples were measured in triplicate, n=4-5.

6.3.3 Effect of ischaemia on baseline and peak $[Ca^{2+}]_i$

Induction of simulated ischaemia on isolated cardiomyocytes resulted in a small decrease in baseline $[Ca^{2+}]_i$ compared with the baseline measurements from cells perfused with normoxic solution (0.78 ± 0.23 vs. 1 F/F_0; Figure 6.6), however this was not a statistically significant effect. Upon stimulation with KCl, normoxic cardiomyocytes exhibited a 1.39 ± 0.14 fold increase in $[Ca^{2+}]_i$ from baseline (Figure 6.7). Under conditions of simulated ischaemia, KCl induced an enhanced fluorescence signal compared to that observed in normoxic cells (2.24 ± 1.46 vs. 1.39 ± 0.14 F/F_0; P=0.05; Figure 6.7).

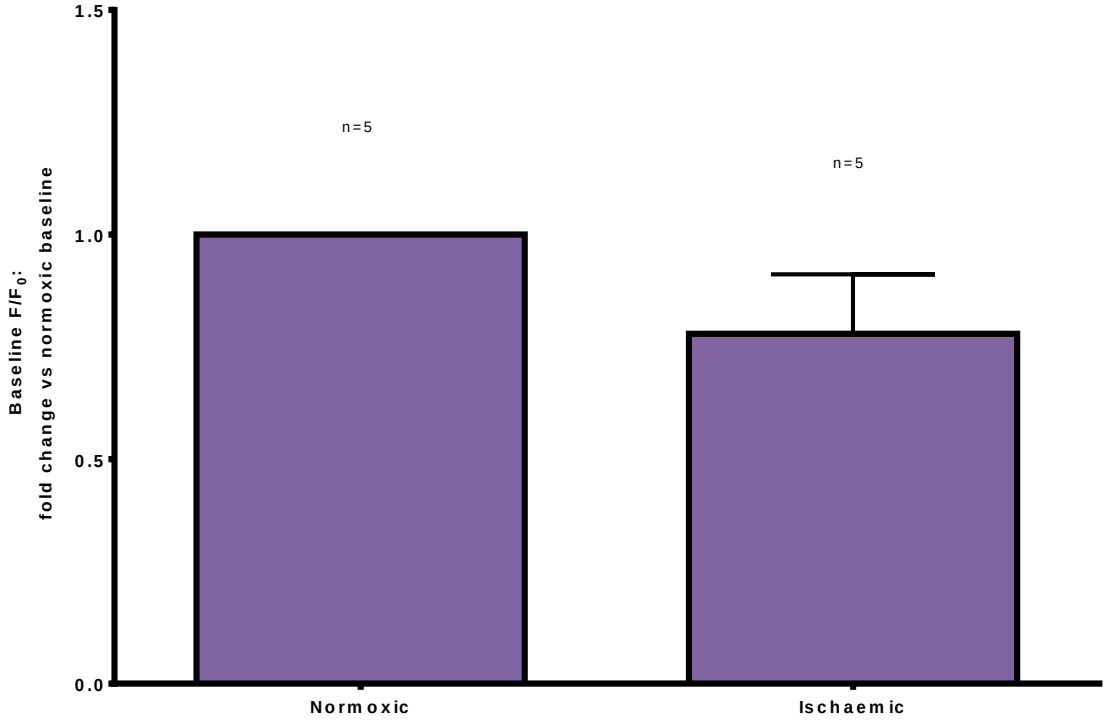

Figure 6.6 Effect of simulated ischaemia on baseline fluo-4 AM fluorescence in isolated cardiomyocytes. Data are expressed as the mean ± SEM fold change from baseline fluorescence in normoxic cells ± SEM (n=3-5).

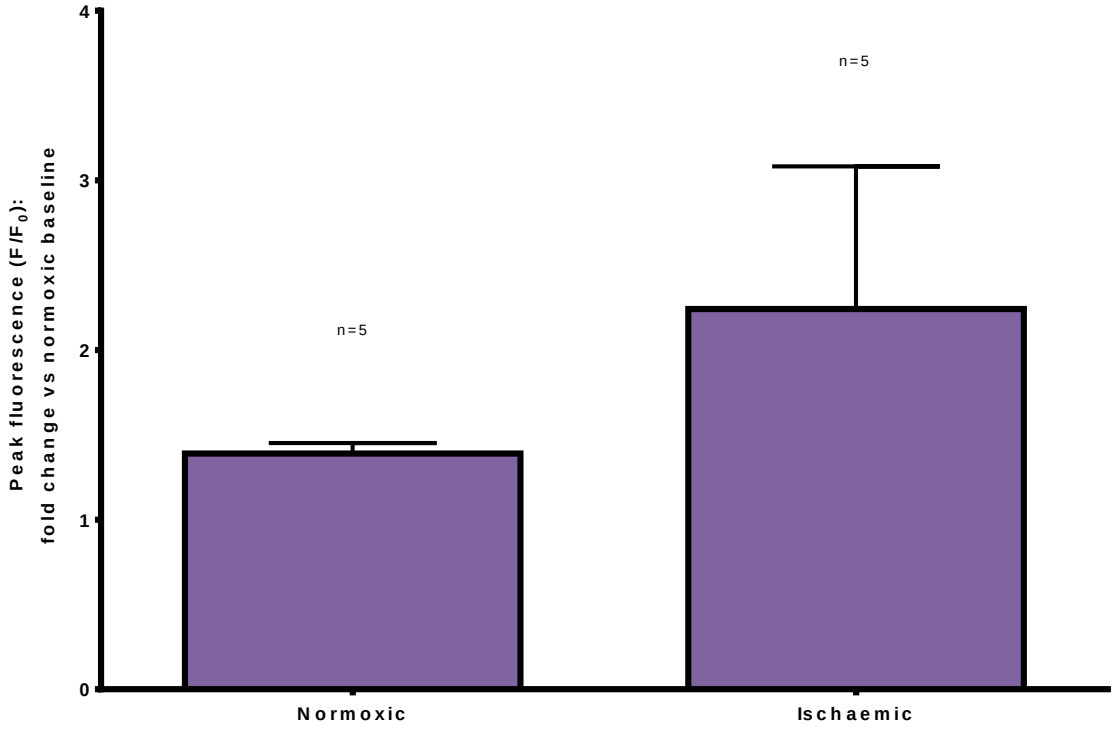

Figure 6.7 Effect of simulated ischaemia on peak fluorescence, elicited by 60mM KCl. Results are expressed as a fold change in mean peak fluorescence (n=3-5).

6.4 Discussion

The aim of this series of experiments was to develop a model of isolation of rodent ventricular cardiomyocytes and to preliminarily determine the effects of CBD and AM251, alone and in combination, on baseline and KCl-stimulated $[Ca^{2+}]_i$ in cardiomyocytes under normoxic and simulated ischaemic conditions.

6.4.1 Method development of isolation of ventricular cardiomyocytes

The principal aim of this series of experiments was to attempt to develop a model to successfully isolate ventricular cardiomyocytes from adult rat hearts. The initial protocol employed (Bates and Gurney, 1999) was modified as described in section 6.2.2 in an effect to improve cell yield and the reproducibility of quality isolations from adult rat hearts. In spite of these modifications, the isolation process remained only partially successful and thus, the preliminary data presented herein represents the effects of simulated ischaemia and drug interventions on $[Ca^{2+}]_i$ on cells whose appearance typified a cardiomyocyte when viewed on an inverted light microscope and which subsequently responded, by a change in fluo 4-AM fluorescence intensity, to application of 60mM KCl (as described in the experimental protocol; figure 6.2).

The protocol (Bates and Gurney, 1999; Ma *et al*., 2006) of enzymatic dispersion of cardiomyocytes from ventricular tissue with collagenase, proteinase and hyaluronidase allows cells to be isolated by digestion of soluble and insoluble collagen, hydrolysis of protein polypeptide chains and cleavage of the glycosaminoglycan, hyaluronan, respectively (Stern and Jedrzejas, 2006). However small changes in tissue levels of the respective targets may mean inadequate or over-digested ventricular tissue. In either circumstance, there would be minimal, if any, functioning cells yielded by enzymatic dispersion. Age-dependent differences in collagen content of cardiac tissues have been demonstrated by Annoni et al. (1998) but not in rats aged less than 12 months. Significant differences in interstitial collagen content were only observed in rats aged 19 months (Annoni *et al*., 1998). As such, it is unlikely that over the duration of the period of the present study, during which rats hearts were harvested for the enzymatic dispersion of cardiomyocytes, that there would have been a significant age-dependent change in cardiac collagen content.

Early isolation of adult rat ventricular cardiomyocytes often produced Ca^{2+}-intolerant cardiomyocytes which were only viable for a few hours post-isolation. The protocols used to yield such cells involved enzymatic dispersion (with collagenase and hyaluronidase or trypsin; Vahouny, 1970; Fabiato and

Fabiato, 1972) of tissue chunks or by retrograde perfusion of the intact heart. It was demonstrated that, in these isolated cells, during the process of returning cells to a bathing medium containing physiological [Ca^{2+}], the cardiomyocytes became metabolically deregulated and this led to hypercontracture, ischaemia and membrane damage (Brierley *et al*., 1985; Thum and Borlak, 2000). Aggressive digestion of cardiomyocytes can lead to membrane damage and may be the cause of Ca^{2+} intolerance, and is typified by a change in the morphology from rod-shaped to spherical and high levels of lactate dehydrogenase (LDH) and creatine kinase (CK) activity. Moreover, these enzymes are good indicators for membrane integrity/damage (Thum and Borlak, 2000). The quantification of viable cells in earlier studies showed a range of viability (55-92%; % of rod-shaped cells) depending on the isolation technique employed with the latter and more successful isolation protocol using an enzyme solution containing Joklik solution supplemented with butandione monoxime (BDM), collagenase type II and bovine serum albumin (Vahouny, 1970; Fabiato and Fabiato, 1972; Grosso *et al*., 1977; Nag and Zak, 1979; Nag *et al*., 1983; Bkaily *et al*., 1984; Van der Heide, 1995; Kivisto, 1995; Weisensee, 1995; Thum and Borlak, 2000). Unfortunately cell viability was not quantified in this study, however from my observations I suspect that the protocol used for this study yielded viability similar to the early adult cardiomyocyte isolation studies. With this in mind, it may be that during isolation in this study, the retrograde perfusion of the heart tissue with the enzyme solutions was too long and the cell membranes became damaged. Thum and Borlak (2000) demonstrated that addition of the cytoprotective agent BDM significantly reduced LDH and CK activity during isolation which may indicate healthier cardiomyocyte cell membranes. As such, it may have been pertinent to include BDM into the perfusion solutions for this study. Improved yield and viability has also been observed with the addition of EDTA early in the isolation process; these improvements were thought to be due to modulation of tight junctions between cardiomyocytes which in turn reduces membrane damage (Thum and Borlak, 2000). Inclusion of these two agents thus may have improved the reproducibility of cell isolation in this study.

This study used a nominally Ca^{2+}-free solution during the enzymatic dispersion of cardiomyocytes by retrograde perfusion as described previously (Bates and Gurney, 1999). Following retrograde perfusion the bathing solution was switched immediately to a Ca^{2+}-containing Krebs solution, which helped to prevent further enzymatic digestion of the cardiomyocytes. More recent investigators have described a more gradual, step-wise replacement of Ca^{2+} into the bathing solution over the course of 20 minutes (Lim *et al*., 2008). This may prevent hypercontracture, ischaemia and membrane damage in cells which may otherwise be lost upon rapid Ca^{2+} replacement (Brierley *et al*., 1985; Thum and Borlak, 2000).

6.4.2 Development of a method to quantify changes in mitochondrial membrane potential

This study initially sought to gain preliminary data of the effect of CBD and AM251 on mitochondrial membrane potential, which was to be determined by a change in fluorescence intensity of the redox sensor red CC-1-loaded cardiomyocytes read on a plate reader and compared with the mitochondrial protonophore FCCP. This was on the premise that CBD and/or AM251 may be conferring cardioprotection (chapter 5) by acting to stabilise mitochondrial membrane potential since AM251 has been shown to attenuate dysfunction of mitochondria in a model of hepatic lipogenesis (Chen, Ho and Lee, 2013) and CBD can modulate $[Ca^{2+}]_i$ depending on the excitability of the cell in a manner in part aided by mitochondria (Ryan *et al*., 2009). Blank and CC-1 loaded cells showed similar fluorescence intensity and moreover, there was no change in fluorescence intensity when mitochondria were uncoupled by any of the concentrations of FCCP tested. It was suspected that a significant number of the isolated cardiomyocytes were being lost during the washing process and so plates were subsequently laminin coated and cells were allowed to adhere for 1, 3 or 5 hours to determine if there was an improvement in fluorescent intensity (appendix 3). This however failed to improve quantifiable fluorescence. Although it was surmised that cardiomyocytes were free-floating and therefore, unaffected by laminin coating of plates, colleagues at Astra Zeneca are currently investigating the physiology of adherent cardiomyocytes using a bind scanner. The difficulty in determining fluorescence intensity using this protocol could therefore be due to low cell yield and as such it was thought pertinent to instead quantify fluorescence using the technique detailed in section 6.2.3, as this protocol is less reliant on high cell yield as the plate reader protocol. In addition, more recently it was shown that AM251 could have a negative impact on mitochondrial membrane potential by modulation of oestrogen-related receptor α (ERRα; Krzysik-Walker *et al*., 2013). With these findings in mind, it was thought that investigation of the effect of CBD and AM251 on $[Ca^{2+}]_i$ may give a better insight into the mechanism by which these agents are anti-arrhythmic, since it is well recognised that modification of $[Ca^{2+}]_i$ reduces arrhythmia. This is in fact thought to be due to reduction in overall cellular Ca^{2+} rather than an effect directly on the mitochondria (Brown and O'Rourke, 2010). Thus, for subsequent experiments the new protocol was employed.

6.4.3 Effect of simulated ischaemia on baseline and KCl-stimulated $[Ca^{2+}]_i$

Fluo-4 AM is a cell permeant fluorescent Ca^{2+} indicator which is excited upon Ca^{2+} binding; as such, an increase in fluo-4 AM fluorescence is indicative of increased intracellular $[Ca^{2+}]_i$.

In conditions of simulated ischaemia there was a slight decrease in baseline fluo-4 AM fluorescence, indicative of a reduction in $[Ca^{2+}]_i$. This is in contrast to findings by Li et al. (2013), who found that simulated ischaemia in cardiomyocytes progressively increased baseline $[Ca^{2+}]_i$. This may be due to the duration of simulated ischaemia in the present study (20 minutes) which was significantly shorter than the 60 minutes used in the Li et al. (2013) study. Similarly, in the *in vivo* setting, induction of ischaemia results in a comparatively slow rise in Ca^{2+} (Levick, 2003).

In control (non drug-treated) cells, upon stimulation with KCl, cardiomyocyte $[Ca^{2+}]_i$ was increased by approximately 1.5-fold under normoxic conditions. When challenged with KCl under simulated ischaemia an approximate 2.5-fold increase in $[Ca^{2+}]_i$ was observed, although this did not achieve statistical significance when compared with normoxic conditions. The increase in $[Ca^{2+}]_i$ observed under conditions of simulated ischaemia were also observed in a more long-term investigation of $[Ca^{2+}]_i$ in a mouse model of chronic heart failure (CHF) after *in vivo* myocardial infarction. Isolated cardiomyocytes from this mouse model of CHF, showed that both 1 week and 10 weeks post-MI, Ca^{2+} transients were higher than in sham animals due to increased Ca^{2+} influx and higher SR Ca^{2+} concentrations, that being Ca^{2+} mobilisation was mediated by electrical field stimulation which was used in the present study as opposed to KCl (Mork *et al*., 2009).

6.4.4 Effects of CBD in normoxic cells on baseline and simulated $[Ca^{2+}]_i$

This study demonstrated that under normoxic conditions, CBD had no effect on baseline $[Ca^{2+}]_i$ in single ventricular cardiomyocytes which is in contrast with findings observed previously in a study of hippocampal cells. Drysdale et al. (2006) showed that CBD (1µM) was a potent mobiliser of $[Ca^{2+}]$, from both intracellular stores and entry via LTCCs, in neurones and glial cells from the hippocampus. However, they did note a degree of variability in the responses to CBD depending on cell type; while 77% of glia responded to CBD, only 55% of neurones did. On the basis of the present findings, it may be that cardiomyocytes are unresponsive to CBD. Furthermore, the study by Drysdale et al. suggests that $[Ca^{2+}]_i$ mobilisation in response to CBD may be CB_1 receptor independent on the basis that the glia responded most frequently but do not express this receptor. As discussed in the introduction, CBD has been proposed to mediate reductions in $[Ca^{2+}]_i$ in conditions of high excitability in hippocampal cells by inhibition of the mitochondrial sodium-calcium exchanger (Ryan *et al*., 2009) and as such, it is unlikely this lack of responsiveness to CBD in our cardiomyocytes is as a result of lack of receptors through which CBD may mediate this reduction in $[Ca^{2+}]_i$.

In contrast to the effect of CBD alone, AM251 pre-treatment unmasked an increase in $[Ca^{2+}]_i$ in response to CBD. This is consistent with the findings of Drysdale et al. (2006), who observed that the

response of hippocampal cells to CBD was markedly increased in the presence of a CB_1 receptor antagonist. They proposed that this CB_1 receptor antagonist-mediated facilitation of the effect of CBD on $[Ca^{2+}]_i$ was due to prevention of voltage-gated Ca^{2+} channel blockade by the tonically active CB_1 receptor. Furthermore, they proposed that CBD's effects were due to release of Ca^{2+} from intracellular stores, rather than Ca^{2+} entry itself, on the basis that the $[Ca^{2+}]_i$ response was blocked by thapsigargin and facilitated by a CB_1 receptor antagonist. Thus, it could be that in the presence of CB_1 receptor blockade CBD is better able to facilitate Ca^{2+} release from intracellular stores, through a possible action at GPR55. However, activation of GPR55 results in Ca^{2+} mobilisation via G_q-facilitated LTCC activation and release of Ca^{2+} from intracellular stores via inositol 1,4,5-triphosphate (IP_3R) and ryanodine (RyR) receptors (Figure 6.13; Lauckner *et al*., 2008; Waldeck-Weiermair *et al*., 2008, Yu *et al*., 2013). Therefore it is more likely that, when free to act as a GPR55 antagonist, CBD inhibits, rather than facilitate, any increase in $[Ca^{2+}]_i$.

6.4.5 Effects of CBD on $[Ca^{2+}]_i$ in simulated ischaemia

Similar to its effects under normoxic conditions, CBD had no effect on $[Ca^{2+}]_i$ under either baseline conditions or in KCl-stimulated cells subjected to simulated ischaemia. Thus it is unlikely that that the anti-arrhythmic effects of CBD described in the previous chapter are through an effect on $[Ca^{2+}]_i$ at the level of the cardiomyocyte.

In contrast to the effect of pre-treatment with AM251 prior to CBD application on KCl-induced increases in $[Ca^{2+}]$ under normoxic conditions, under conditions of simulated ischaemia, co-application of AM251 suppressed the rise in $[Ca^{2+}]_i$ suggesting that ischaemia *per se* induces changes that leads to a reduction in the $[Ca^{2+}]_i$ response. Levels of circulating endocannabinoids have been shown to increase after induction of acute ischaemia and AEA has been shown to be released directly from cardiomyocytes (Mukhopadhyay *et al*., 2007) and to inhibit the function of LTCCs. Thus, under conditions of ischaemia AEA released from the cardiomyocytes may inhibit the KCl-stimulated rise in $[Ca^{2+}]_i$ through blockade of LTCCs, thus negating the effect of any CBD-mediated rise in $[Ca^{2+}]_i$.

6.4.6 Effects of AM251 on baseline and stimulated $[Ca^{2+}]_i$ in normoxic and hypoxic cardiomyocytes

AM251 alone induced a significant increase in both baseline and KCl-induced increases in $[Ca^{2+}]_i$ in normoxic, isolated cardiomyocytes, which is consistent with a previous study demonstrating that AM251 increases Ca^{2+} currents in HEK 293 cells (Vasquez *et al*., 2003). If the effects of AM251 are

through its action as a CB_1 antagonist, then this increase in $[Ca^{2+}]_i$ may be due to lifting the CB_1-mediated inhibition of GPR55 signalling as described above. However, CB_1 receptors are expressed only sparsely on normal, healthy cardiomyocytes, which suggests that in normoxic cells the actions of AM251 are likely to be independent of CB_1 receptors, and thus more likely involve the activation of GPR55 which, would increase internal Ca^{2+} (Lepicier *et al*., 2007). As mentioned above, GPR55 activation in cardiomyocytes has been shown to regulate $[Ca^{2+}]_i$ in a L-type Ca^{2+} channel, IP_3R and RyR-dependent manner (Henstridge *et al*., 2009; Yu *et al*., 2013). In addition, GPR55 receptor activation can induce depolarisation of the cardiomyocyte (Bondarenko *et al*., 2010; Yu *et al*., 2013). Importantly, GPR55 receptor activation can induce different electrophysiological responses depending on whether activation is sarcolemmal or intracellular, with the former depolarising and the latter hyperpolarising the cardiomyocyte (**Error! Reference source not found.**6.16; Yu *et al*., 2013). The finding that the increases in baseline and peak $[Ca^{2+}]_i$ elicited by AM251 were attenuated when cells were pre-treated with CBD prior to AM251 supports the notion of an action of AM251 at GPR55.

In contrast to the effects on normoxic cardiomyocytes, AM251 decreased the KCl-induced increase in $[Ca^{2+}]_i$ and had no effect on baseline $[Ca^{2+}]_i$. Moreover, the effect on KCl-induced changes in $[Ca^{2+}]_i$ was not modulated by CBD pre-treatment. This suggests once again that ischaemia can alter the response of cardiomyocyte $[Ca^{2+}]_i$ to AM251. As discussed in section 6.4.3, the most likely mediator of reduced $[Ca^{2+}]_i$ in conditions of ischaemia would be increased circulating endocannabinoids that inhibit Ca^{2+} influx through the LTCCs (Mukhopadhyay *et al*., 2007). Alternatively, hypoxia/ischaemia may influence the expression of the receptors within the myocardium, since the expression of both CB_1 (Lepicier *et al*., 2007) and GPR55 (chapter 5) have both been shown to be augmented in ischaemia/infarcted myocardium; this would inevitably result in alterations in the pharmacological response to agents acting at these receptors. More specifically, data from our lab has shown that LPI, the endogenous ligand for GPR55, can only increase infarct size when given prior to ischaemia and not post-MI (Robertson-Gray, unpublished). This may suggest that ischaemia is associated with a reduction in GPR55 expression and this may explain the opposing effects of AM251 in the isolated cardiomyocytes.

6.4.7 Study limitations

The model of simulated ischaemia in isolated ventricular cardiomyocytes used in the present study is similar to that implemented by O'Brien and Howlett (2008). While is does not mimic precisely the effects of *in vivo* MI, the purpose of this study was to further elucidate the anti-arrhythmic effects of CBD and AM251 and more specifically, whether they might be acting to alter $[Ca^{2+}]_i$. As such

investigation of the isolated cardiomyocyte under conditions of hypoxia, substrate deprivation, and ionic imbalance was a fair representation of *in vivo* conditions (Guo *et al*., 2013).

Acute *in vivo* ischaemia induced by ligation of a coronary artery causes cessation of local circulation, thus inducing oxygen deprivation and metabolite accumulation. In the *in vitro* model of simulated ischaemia used in this study, an ischaemic bath solution was superfused to the cardiomyocyte perfusion chamber. Therefore, in this model, there was no cessation of circulation as would be observed *in vivo*. Critically, purine precursors, amphiphiles and protons would be washed out, thereby preventing replication of the deleterious effects of metabolite accumulation (Carmeliet, 1999).

In conditions of acute hypoxia, there are changes in ion channel function which consequently induce ion concentration perturbations, including but not limited to; the late Na^+ which is amplified, the fast Na^+ which is reduced, the L-type Ca^{2+} current through which basal current is diminished, and finally, the delayed rectifier K^+ current, through which the slow component is decreased. The perturbations in ion homeostasis change action potential morphology duration little unless coupled with β-adrenoceptor stimulation (Guar, Rudy and Hool, 2009). The changes in ion channel function following acute hypoxia are similar to those induced by ischaemia, although since action potential morphology is altered less, the ion perturbations are less extreme. In hypoxia, the action potential is shortened as in conditions of coronary artery occlusion but the fall in resting membrane potential is not as great, furthermore, the maximum upstroke velocity decreases are not as significant (McDonald and MacLeod, 1973; Carmeliet, 1978; Janse and Wit, 1989; Pacini and Kane, 1991; Carmeliet, 1999). It has been identified that a combination of lactate (10mM), acidosis (pH 6.8) and the bioactive lipid, α-lysophosphatidylcholine (LPC; 5µM), most closely reflected the changes in action potential observed *in vivo*, however, these *in vitro* changes were best observed in what would be the late phase of arrhythmia occurrence *in vivo* (>6 hours post-occlusion). The changes in AP characteristics induced by combined acidosis, hypoxia and elevated extracellular K^+, conditions we employed, produced reliable reductions in resting membrane potential, action potential amplitude and action potential duration at 50 and 90% repolarisation levels (Pacini and Kane, 1991) thus supporting the use of these conditions in our model.

The model described herein cannot therefore completely represent the plethora of deleterious changes induced by acute MI *in vivo*. However the hypoxic conditions, combined with an ischaemia-like superfusate, are a viable representation in the isolated, *ex vivo* setting.

6.4.8 Summary and conclusions

The key findings are that, CBD has no significant effect on $[Ca^{2+}]_i$ in normoxia or simulated ischaemia, however, AM251 pre-treatment unmasked an increase in $[Ca^{2+}]_i$, possibly induced by activation of SR release or by GPR55-mediated activation by AM251. AM251 however, increased baseline $[Ca^{2+}]_i$, likely due to GPR55 activation as this response could be muted by CBD pre-treatment, implying a simple, agonist-antagonist effect of the two compounds at GPR55. AM251 either alone or in combination with CBD reduced $[Ca^{2+}]_i$ in hypoxic cardiomyocytes and this may explain the anti-arrhythmic effects of this drug we described in chapter 5.

7. General Discussion

7.1 Main findings

7.1.1 CBD and AM251 mediate hypotension in the anaesthetised rat

The role that CB_1 receptors play in mediating changes in haemodynamic variables has been reasonably well characterised, having been identified as the principal mediators of the prolonged hypotension which typifies phase III of the haemodynamic response to bolus I.V. cannabinoid administration (Jarai *et al*., 1999; Ledent *et al*., 1999). As detailed in sections 4.4.1 and 4.4.2, this study further supports these data by demonstrating that the selective CB_1 receptor agonist, ACEA, produces a significant fall in MABP following bolus I.V. administration. Furthermore, the CB_1 receptor antagonist AM251 had the capacity to abrogate the depressor response induced by ACEA, thus strongly suggesting that the effects of ACEA were mediated by the CB_1 receptors.

Unlike the role of CB_1 receptors in blood pressure regulation, the role of GPR55 has received much less attention, yet increasing evidence suggests that this recently deorphanised GPCR may play an important physiological role. In particular it has been identified to play a role in bone metabolism (Whyte *et al*., 2009; Sharir *et al*., 2012), skin tumour development (Perez-Gomez *et al*., 2013), and the prevention of oxidative damage and neutrophil migration (McHugh and Ross, 2009; Balenga *et al*., 2011; Henstridge *et al*., 2011). Alone it was observed that AM251 induced a transient depressor response which could not be explained as a CB_1 receptor mediated effect given that antagonism at CB_1 would be expected to increase, rather than decrease, blood pressure (Montecucco and Di Marzo, 2012). As well as being a CB_1 antagonist however, AM251 is a potent GPR55 receptor agonist (Ryberg *et al*., 2007), and as such, it is possible that the transient fall in MABP induced by AM251 is a GPR55-dependent effect.

In addition to the haemodynamic changes mediated by each of these agents alone, pre-treatment with CBD abrogated the AM251 mediated antagonism of ACEA's depressor effects. CBD (Ryberg *et al*., 2007) and AM251 (Fischbach et al., 2007; Johns *et al*., 2007; Waldeck-Weiermair *et al*., 2007) have opposing pharmacological activity at GPR55, as antagonist and agonist, respectively, and as such it would be unlikely that this abrogation is solely through a GPR55-mediated effect. Rather, literature exists which suggests that there is a degree of crosstalk between the CB_1 and GPR55 receptors with GPR55 signalling being inhibited by CB_1 receptor activation in a process dependent on the activation status of integrins (Waldeck-Weiermair *et al*., 2008). Therefore if this cross-talk is relevant to modulation of haemodynamic variables then it is possible that CBD is not acting as an antagonist at

GPR55 but rather as a weak antagonist or inverse agonist at CB_1, thus uplifting CB_1-mediated suppression of GPR55 signalling thus allowing AM251 to induce a more profound hypotension.

7.1.2 The selective GPR55 agonist, O-1602, does not mediate hypotension in the anaesthetised rat

To confirm that the transient hypotension mediated by AM251 was a GPR55-medited effect the selective GPR55 agonist, O-1602 was employed; however, this failed to induce any change in MABP or HR, even at the highest dose (100ng kg^{-1}) as described in sections 4.4.5. Recent data has however, cast doubt over the legitimacy of O-1602 as a GPR55 ligand and suggestion was made that rather that GPR55, GPR18 is its principal pharmacological target (Johns *et al*., 2009; Kapur *et al*., 2009; McHugh and Ross, 2009). Thus the lack of O-1602 induced hypotension in the anaesthetised rat does not rule out a role for GPR55 in BP regulation.

7.1.3 Pre-ischaemia bolus AM251 administration confers protection against ischaemia-induced ventricular arrhythmias *in vivo*

Numerous studies exist which investigate the effects of cannabinoids in the cardiovascular system including examination of the negative ionotropy induced by CB_1 receptor activation (Ford *et al*., 2002; Bonz *et al*., 2003) and vasodilator effects of the endocannabinoid AEA (O'Sullivan *et al*., 2004; Mukhopadhyay *et al*., 2002). More recently our lab identified the cardioprotective effects of the phytocannabinoid, CBD (Walsh *et al*., 2010). Thus the aim of the present study was to elucidate the targets which may confer this cardioprotection.

This study has, for the first time, demonstrated the anti-arrhythmic effect of the CB_1 receptor antagonist and GPR55 receptor agonist, AM251 in a rat model of acute MI. Although this contrasts with existing literature (Hajrasouliha *et al*., 2008), this may be explained by the fact that the VT incidence in their control animals was comparable in magnitude to that observed for our AM251-treated animals and therefore, much lower than our control incidences. As detailed in section 5.5.2, it was initially postulated that the anti-arrhythmic effects of AM251 may be conferred by way of compensatory activation of CB_2 receptors owing to the inhibition of CB_1 receptors coupled with the possibility that CB_1 receptor expression is reduced during periods of increased endocannabinoid tone, which in the setting of ischaemia, would persist (Mukhopadhyay *et al*., 2007; Nucci *et al*., 2007). The role of CB_2 receptor activation in cardioprotection has been more thoroughly investigated than that of

CB_1, and it is acknowledged to provide protection (Krylatov *et al*., 2001; Lucsokova *et al*., 2008; Lim *et al*., 2009). Interestingly however, the present study demonstrated that AM251 had a marked suppressant effect on arrhythmias during acute MI which were likely to be induced by excess catecholamine release (i.e. phase Ib). One possibility is that AM251 achieves this through inhibition of tyrosine hydroxylase expression, which it has already been demonstrated to reduce in fawn-hooded rats (Femenia *et al*., 2010). Tyrosine hydroxylase is a critical catalyst of the rate limiting step in the production of adrenaline and noradrenaline (Femenia *et al*., 2010).

7.1.4 Co-administration of CBD and AM251 can potentiate the reduction in arrhythmia incidence of either alone

In order to identify potential targets which may mediate the anti-arrhythmic effects of CBD (Walsh *et al*., 2010), the effect of CBD in the presence of AM251, which was initially used as a CB_1 antagonist, was investigated. During these investigations it became clear than AM251 alone was also able to reduce the incidence of VT and total VPBs and furthermore, depending on the order of administration, co-administration of CBD and AM251 could potentiate the observed reductions in VT incidence and total VPBs compared when either agent is administered alone. The disparity in the degree of protection afforded by CBD and AM251 depending on the order of administration, points to the involvement of other receptors, than just the CB_1 receptor. Therefore it is possible that there is a degree of synergism between CBD and AM251 at common receptor targets, and this is supported by unpublished observations from our laboratory that have demonstrated that the modest *ex vivo* anti-aggregatory capacity of CBD is enhanced in the presence of AM251. The common receptor targets may include the putative cannabinoid receptor GPR55. CBD and AM251 both act at GPR55, as an antagonist and agonist, respectively, moreover, CB_1 receptors and GPR55 receptors have been shown to interact *in vitro* (Lauckner *et al*., 2008; Pacher and Hasko, 2008; Waldeck-Weiermair *et al*., 2008; Lim *et al*., 2009). However, while it seems unlikely that this synergism is the means via which AM251 and CBD confer an anti-arrhythmic effect, considering the individual actions of these compounds at these receptors the difference in their anti-arrhythmic capacity based on order of administration may simply be due to a preferential activation of these targets based on their pharmacologies as detailed in figures 5.21 and 5.22.

7.1.5 AM251 can regulate $[Ca^{2+}]_i$ in cardiomyocytes

As detailed in section 6.3.4 and 6.3.5, AM251 can increase $[Ca^{2+}]_i$ in baseline and stimulated normoxic cardiomyocytes. Although AM251 was initially employed as a CB_1 receptor antagonist in the haemodynamic study (Chapter 3) it is now evident that AM251 is not a selective antagonist for CB_1 but can in addition, act as an agonist at GPR55 (Ryberg *et al*., 2007). The present data supported observations made by Li et al. (2013) and since GPR55 receptor activation can increase $[Ca^{2+}]_i$ by mechanisms including LTCC activation and IP_3R and RyR mediated release from intracellular stores (Yu *et al*., 2013), it seems possible that the increase in $[Ca^{2+}]_i$ mediated by AM251 is due to activation of GPR55.

Conversely, AM251 appeared to induce a decrease in $[Ca^{2+}]_i$ in stimulated, cardiomyocytes under ischaemic conditions. This effect was unaltered when CBD was co-administered. The data therefore suggested that ischaemia itself was altering the response of AM251 on $[Ca^{2+}]_i$ in cardiomyocytes. Furthermore, our immunohistochemical identification of GPR55 in heart tissue (described in chapter 5) shows that GPR55 receptor expression may be changed following ischaemia and thus, AM251 may no longer be acting predominantly at GPR55 but rather as an antagonist at CB_1 receptors which are tonically active (Romano *et al*., 2013).

7.1.6 CBD does not mediate changes in $[Ca^{2+}]_i$ in cardiomyocytes

CBD had no effect on $[Ca^{2+}]_i$ in baseline or stimulated cardiomyocytes, under either normoxic or hypoxic conditions. This contrasted with data from Drysdale et al. (2006), who found CBD to be a potent mobiliser of Ca^{2+} in neurones and glia. However, these authors noted that there was a degree of variability in responses to CBD depending on the cell type and thus, it may be that myocytes are relatively unresponsive to CBD. On the basis that CBD does not affect a change in $[Ca^{2+}]_i$ in myocytes under hypoxic conditions, it seems unlikely that the current findings and the previously reported anti-arrhythmic effects of CBD are mediated by a change in $[Ca^{2+}]_i$ at the level of the cardiomyocyte (Walsh *et al*., 2010).

Pre-treatment with AM251 unmasked an ability of CBD to increase baseline and stimulated $[Ca^{2+}]_i$ in normoxic cells. This data supports previous observations which noted that the response of hippocampal cells to CBD was increased in the presence of a CB_1 receptor antagonist (Drysdale *et al*., 2006). These authors proposed that the facilitation of $[Ca^{2+}]_i$ mobilisation was due to relief of voltage-gated Ca^{2+} channel blockade by the tonically active CB_1 receptor. I also considered the potential

involvement of GPR55 given the pharmacological profiles of CBD and AM251 at this receptor. GPR55 activation is acknowledged to mediate an increase in $[Ca^{2+}]_i$ and thus, the increase in $[Ca^{2+}]_i$ conferred by CBD and AM251 and not CBD alone may be due to activation of GPR55 by AM251. However, CBD and AM251 did not increase $[Ca^{2+}]_i$ in conditions of hypoxia, in fact there was a trend toward a reduction in $[Ca^{2+}]_i$ in conditions of hypoxia. This may suggest that hypoxia itself could be inducing a change which may affect $[Ca^{2+}]_i$ at the level of the cardiomyocyte. Recently, our group has shown that LPI can increase infarct size when given pre- but not post-ischaemia which would support the hypothesis that GPR55 activity is modulated by ischaemia (Robertson-Gray, unpublished).

7.1.7 Clinical relevance

This study has demonstrated the beneficial role of cannabinoid and GPR55 receptor ligands in reducing ischaemia-induced ventricular arrhythmias and furthermore, regulating $[Ca^{2+}]_i$ in conditions associated with hypoxia. In the clinical setting MI is the cause of angina and cardiac arrhythmias (Crossman, 2004). Prolonged periods of ischaemia can cause cardiomyocyte death by myocardial infarction which is defined by ST, T and QRS complex changes on an ECG and an increase in blood concentrations of specific biomarkers like cardiac troponin. Acute coronary events like myocardial infarction continue to have a high mortality rate due to life-threatening in the first couple of hours and furthermore, fatalities in the first month can range from 30-50 % (Armstrong *et al*., 1972; Tunstall-Pedoe *et al*., 1999; Van de Werf *et al*., 2003). Although these studies focussed on the pre-ischaemic administration of CBD and AM251 and thus this treatment would not be clinically practical for myocardial infarction or other acute coronary events, it may be practical for use on occasions which necessitate cessation of normal circulatory function, for example, coronary artery bypass grafts during which ischaemia-reperfusion injury routinely occurs (Sabbagh *et al*., 2013).

7.2 Future work

7.2.1 The role of GPR55 in regulating $[Ca^{2+}]_i$

The current literature and this data suggest a role for the GPR55 receptor in regulating $[Ca^{2+}]_i$ at the level of the cardiomyocyte in conditions of normoxia, and perhaps also in hypoxic conditions, as such and due to the preliminary nature of the isolated cardiomyocyte data presented in this thesis it would be interesting to repeat the investigations of the effects of AM251 in $GPR55^{-/-}$ cardiomyocytes to

precisely determine the role this receptor may be playing. Furthermore, it would be interesting to determine if the effects of AM251 are due to selective GPR55 activation or whether its ability to act at CB_1 receptors also plays a role. Recently, some groups have identified novel GPR55 ligands which lack activity at CB_1 and CB_2 and so use of these could possibly indicate if CB_1 has any involvement or not (Brown *et al*., 2011; Kotsikorou *et al*., 2011; Kargl *et al*., 2012).

7.2.2 Cross-talk between CB_1 and GPR55

It has been recognised that the activation and consequent signalling cascades of CB_1 and GPR55 receptors are linked (Waldeck-Weiermair *et al*., 2008; Kargl *et al*., 2012). Kargl et al. (2012) showed that GPR55 and CB_1 receptors can form heteromers and that this process can modulate the signalling properties of each receptor. Therefore, it would be interesting to investigate whether the responses to CBD and AM251 are similar in cells expressing each receptor alone and those expressing the heteromeric receptors to determine if a difference exists between the responses. Moreover, if these responses were compared in isolated cardiomyocytes in particular they would give an indication as the whether 'cross-talk' is involved in the changes previously observed in $[Ca^{2+}]_i$.

7.2.3 Examination of the effect of GPR55 on ischaemia-induced ventricular arrhythmias

Our lab has previously sought to investigate the role of GPR55 receptors in a mouse model of acute MI; however the effect of AM251 on ischaemia-induced arrhythmias was not quantified. To better understand the involvement of GPR55 and CB_1 receptors in mediating the cardioprotective effects of AM251 and CBD, it would be interesting to repeat this study in the $GPR55^{-/-}$ model

7.2.4 Determination of the effect of AM251 and CBD on infarct size

It would be interesting to identify whether the effects of CBD on ventricular arrhythmias extend to myocardial necrosis. As such it would be beneficial to investigate the effect of AM251 and CBD on infarct size.

7.3 Conclusions

This study has shown that both the CB_1 receptor and GPR55 have the potential to induce haemodynamic changes despite the complex nature of ligand specificity encountered. Moreover, it has been demonstrated that, while both CBD and AM251 compounds have the capacity to reduce ischaemia-induced ventricular arrhythmias, they probably mediate these changes at distinct sites. In particular, the cardioprotective effects of CBD do not appear to be mediated by a change in $[Ca^{2+}]_i$ at the level of the cardiomyocyte. In contrast, the anti-arrhythmic effects of AM251 are unlikely due to an effect on $[Ca^{2+}]_i$. This data suggests a role for the GPR55 receptor in mediating changes in Ca^{2+} and also in reducing ischaemia-induced ventricular arrhythmias and thus, it may represent a promising new target for ameliorating the pathological effects of cardiovascular disease.

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APPENDIX I

Materials list

ACEA: purchased pre-dissolved in ethanol (EtOH), this was evaporated off in a steady stream of nitrogen, before being re-solubilised in tween 80, DMSO and NaCl (to the ratio 1:2:8, respectively) to a concentration of 3mg ml^{-1}. Tocris Bioscience, Bristol, UK,

ADP: Diluted in saline to a concentration to 5µM. Chrono-log, Havertown, USA,

AKT1 primary antibody (rabbit polyclonal; phospho S473): Was diluted in TBS. Abcam, Cambridge, UK,

AM251: solubilised in tween 80, DMSO and NaCl (to the ratio 1:2:8, respectively. Tocris Bioscience, Bristol, UK,

Anandamide (AEA): pre-dissolved in EtOH to a concentration 5mg ml^{-1}. Diluted for experimental purposes, as required. Tocris Bioscience, Bristol, UK,

Background Sniper: ready-to-use blocking reagent. Biocare Medical, Concord, USA,

Betazoid DAB chromogen kit: prepared as per instructions with the DAB chromogen kit. Biocare Medical, Concord, USA,

CBD: solubilised in EtOH. Tocris Bioscience, Bristol, UK,

CB_1 receptor primary antibody (rabbit polyclonal): Was diluted in TBS. Abcam, Cambridge, UK,

Collagenase (Worthington class; Type II from *Clostridium histolyticum*): solubilised in nominally-free Ca^{2+} buffer solution. Sigma-Aldrich. Gillingham, UK,

DaVinci Green Dilutent: universal dilutent for GPR55 dissolution. Biocare Medical, Concord, USA,

DIVA decloaker (10x): ready-to-use buffer for antigen unmasking. HistoLab Products AB, Gothenburg, Sweden,

Evans Blue dye: solubilised in distilled water. Sigma-Aldrich, Gillingham, UK,

Fluo-4AM: re-constituted in 100% DMSO to a concentration of 5mM. Diluted in to the appropriate working concentration in micro-centrifuge tubes containing 1ml cell suspension. Molecular Probes Invitrogen, Paisley, UK,

Formal fixx: A ready-to-use 10% neutral buffered formalin solution. Thermo Fisher Scientific, Leicestershire, UK,

GPR55 primary antibody (rabbit polyclonal; LS-A6817 and LS-A162): Diluted in DaVinci Green Dilutent. MBL International Corporation, Massachusetts, USA,

Haematoxylin Harris': Ready-to-use solution. Sigma-Aldrich, Gillingham, UK,

Heparinised saline: Diluted in 0.9% NaCl. Leo Laboratories Buckinghamshire, UK,

Hyaluronidase (Type I-S; from bovine testes): Solubilised in nominally-free Ca^{2+} buffer solution. Sigma-Aldrich, Gillingham, UK,

Hydrogen peroxide (H_2O_2): Diluted with distilled water as appropriate. Sigma-Aldrich, Gillingham, UK,

ImmPRESS reagent: ready-to-use solution provided with the ImmPRESS universal antibody (anti-mouse Ig/anti-rabbit Ig, peroxidase) polymer detection kit. Vector Labs, Burlingame, USA,

Immu-mount: An aqueous, non-fluorescing mounting media. Anatomical Pathology International, Cheshire, UK,

KCl: solubilised in distilled water to a stock concentration of 2M,

MACH 3 rabbit HRP detection kit: pre-prepared two-step polymer detection kit. Biocare Medical, Concord, USA,

Mountex Mounting Medium: Histoab, Sweden,

Noradrenaline: re-constituted in 0.5M HCl to a concentration of 50mg ml^{-1}, with working stocks of 0.1µM,

Normal horse blocking serum (2.5%): ready-to-use solution provided with the ImmPRESS universal antibody (anti-mouse Ig/anti-rabbit Ig, peroxidase) polymer detection kit. Vector Labs, Burlingame, USA,

O-1602: solubilised in 100% methyl acetate to a working concentration of 10mg ml^{-1}. Working stock concentration of 30ng ml^{-1} prepared from serial dilutions in saline of 100µg ml^{-1} to 1000ng ml^{-1}. Tocris Bioscience, Bristol, UK,

Pentobarbital sodium salt: solubilised in NaCl to a concentration of 60mg ml^{-1} on a daily basis. Sigma-Aldrich, Gillingham, UK,

Periodic acid: ready-to-use 1% solution provided with the periodic acid-Schiff (PAS) kit. Sigma-Aldrich, Gillingham, UK,

Peroxidazed 1: ready-to-use blocking reagent. Biocare Medical, Concord, USA,

Phosphate-buffered saline (PBS): 1 PBS tablet dissolved in 100 ml of distilled water. Final solution composition; 137mM NaCl, 10mM phosphate, 2.7mM KCl: pH 7.4. Sigma-Aldrich, Gillingham, UK,

Proteinase (Type XXIV; bacterial): dissoluted in nominally-free Ca^{2+} buffer solution. Sigma-Aldrich, Gillingham, UK,

Rabbit IgG: reconstituted in 1ml of distilled water. Diluted in TBS as to be Eqimolar with the primary antibody. Vector Laboratories, Burlingame, USA,

Saline: 0.9% NaCl in distilled water,

Schiff's reagent: ready-to-use solution provided with the periodic acid-schiff (PAS) kit. Sigma-Aldrich, Gillingham, UK,

Tachas Haematoxylin: Biocare Medical, Concord, USA,

Taurine: dissoluted in nominally-free Ca^{2+} buffer solution. Sigma-Aldrich, Gillingham, UK,

TBS automation wash buffer (20X): diluted as per manufacturer's instructions. Biocare Medical, Concord, USA,

TBS plus 0.025% triton X-100: triton x-100 dissoluted in TBS,

Toluidine blue (0.1% w/v): 1% toluidine blue top stock prepared in 70% ethanol. Working stock of 0.1% prepared in saline. Thermo Fisher Scientific, Leicestershire, UK,

Triphenyltetrazonium chloride (TTC): solubilised in distilled water. Sigma-Aldrich, Gillingham, UK,

URB 597: solubilised in EtOH and diluted as appropriate. Enzo Life Sciences, Exeter, UK,

VectaMount: Vector Labs, Burlingame, USA,

Vector VIP: prepared as per protocol provided with the VIP substrate kit for peroxidase. Vector Labs, Burlingame, USA,

Verapamil: re-constituted in distilled water. Sigma-Aldrich, Gillingham, UK,

2-arachidonylglycerol: solubilised in EtOH. Tocris Bioscience, Bristol, UK.

APPENDIX II

Cardiomyocyte isolation

Original protocol for the preparation of cell extracts from adult rat ventricular myocytes

After terminal anaesthesia with 2ml sodium pentobarbital sodium salt (Sigma-Aldrich), hearts were excised and the aorta cannulated before being mounted on the Langendorff apparatus (as described by Bates and Gurney, 1999). The hearts were then subjected to retrograde perfusion, maintained at 12ml ml^{-1} and 37°C, with a nominally Ca^{2+}-free buffer solution (Solution A). This ensured that blood was washed from the coronary vasculature. Solution A contained (in mM): 120 NaCl, 5.4 KCl, 5 $MgSO_4$, 5 $C_3H_3NaO_3$, 20 glucose, 20 taurine and 10 HEPES (pH 6.96). After 5 minutes of perfusion with solution A, perfusion was continued with solution A containing 4U ml^{-1} proteinase (sigma type XXIV) for 2 minutes. The final perfusate was solution A, containing 0.3mg ml^{-1} collagenase (Worthington class 2) and 0.6 mg ml^{-1} hyaluronidase for 5-10 minutes. Following completion of perfusion, hearts were dismounted from the Langendorff apparatus, atria cut from the ventricles and discarded. The ventricular tissue was then finely minced in warm KB solution, which contained (in mM): 30 KCl, 3 $MgSO_4$, 30 KH_2PO_4, 10 glucose, 20 taurine, 10 HEPES, 85 KOH, 50 glutamic acid and 0.5 EGTA (pH 7.4). The minced tissue was then centrifuged briefly, re-suspended in solution A, if being used immediately, or KB solution, if being stored in the fridge overnight.

APPENDIX III

Optimisation of cell adherence of isolated ventricular cardiomyocytes

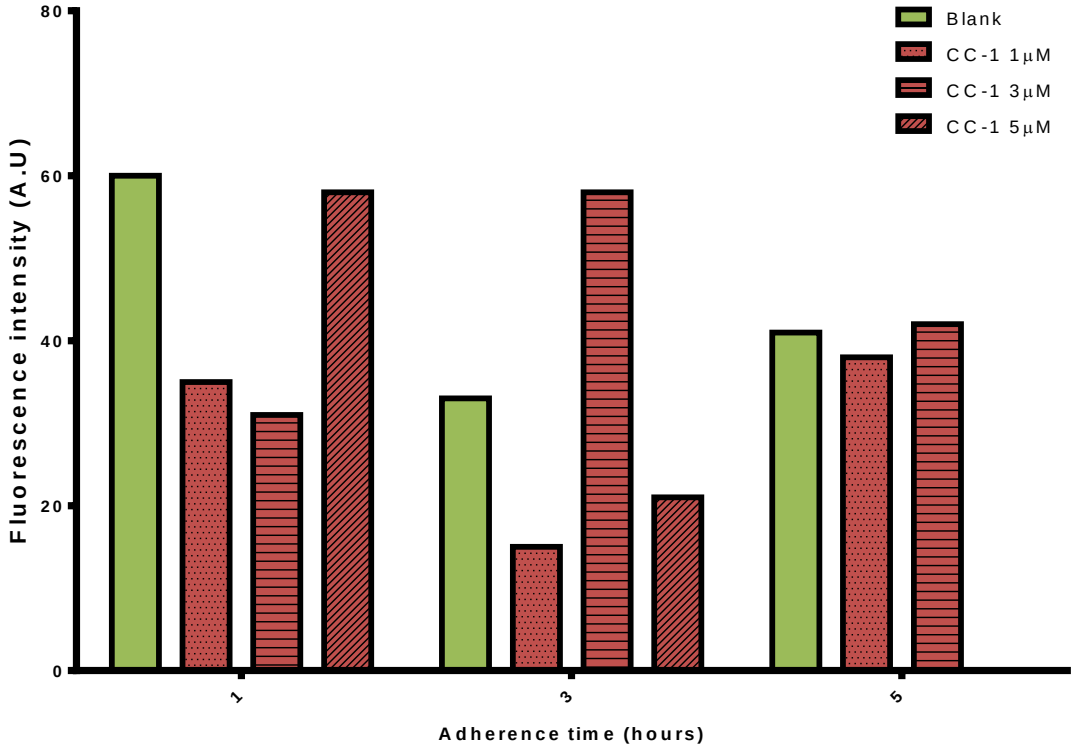

Figure III.I The effect of extended adherence of ventricular cardiomyocytes on fluorescence intensity. Samples were measured in triplicate (n=1).

A range of adherence times were investigated in order to determine if fluorescence intensity in isolated cardiomyocytes could be improved by cardiomyocytes being adhered to laminin-coated plates prior to incubation with CC-1. Although this experiment was only performed on an n of 1, there appeared to be no benefit to allowing cells longer to adhere before commencing the experimental protocol.