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An investigation of cannabinoid-mediated cardioprotection

A thesis submitted to Cardiff University for the degree of Doctorate of Philosophy

Nichola Joy Underdown

Welsh School of Pharmacy

Cardiff University

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Publications

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Underdown, N.J., Hiley, C.R., & Ford, W.R. (2004). Dimethyl sulfoxide inhibits cannabinoid-mediated infarct size limitation in rat isolated hearts. Proceedings of the British Pharmacological Society at http://www.pa2online.org/Vol2Issue4abst046P.html

Underdown, N.J., & Ford, W.R. (2004). Anandamide reduces infarct size in rat isolated hearts by a mechanism independent of CB_1 or CB_2 receptor activation. Proceedings of the British Pharmacological Society at http://www.pa2online.org/Vol2Issue2abst091P.html

Underdown, N.J., & Ford, W.R. (2003). Mechanism of Anandamide-induced positive chronotropy in rat isolated right atria. Proceedings of the British Pharmacological Society at http://www.pa2online.org/Vol1Issue4abst161P.html

Summary

This thesis describes work carried out to characterise cardiac responses to cannabinoids. A particular focus of the work was to study the mechanism by which cannabinoids protect hearts from ischaemia-reperfusion injury

Studied in rat isolated right atria, anandamide (an endocannabinoid) induced a limited positive chronotropy. As this response was blocked by indomethacin and not mimicked by methanandamide (a non-hydrolysable analogue), the mechanism of action appears to involve conversion and release of products of cyclooxygenase-2. Conversely, negative chronotropy was observed when baseline rate was elevated by a ß-adrenoceptor agonist. The pharmacological profile of this anti-adrenergic response indicated the involvement of CB_2 receptors.

Potential protection afforded by cannabinoids against rat isolated hearts subjected to ischaemia-reperfusion was studied in two different models. In the first, hearts were subjected to global, no-flow ischaemia-reperfusion with agonists introduced 5 min before ischaemia. The second method subjected hearts to regional ischaemia-reperfusion with agonists introduced 35 min into ischaemia. Antagonists, where used, were present throughout the protocol.

In the global, no-flow ischaemia-reperfusion model both anandamide and methanandamide significantly reduced infarction but in the regional ischaemia-reperfusion model only methanandamide significantly reduced infarction. In both models the infarct limiting action was lost in the presence of both the CB_1 receptor

antagonist SR141716A and the CB_2 receptor antagonist SR144528. However, CB_1- or CB_2-receptor agonists, used alone or in combination, were ineffective at reducing infarct size. These results suggest that neither CB_1 nor CB_2 receptors, alone or in synergy mediate cardioprotection. These results suggest that one or more novel sites of action, possibly a new cannabinoid receptor subtype, might mediate cardio-protection in rat hearts.

These findings demonstrate the potential for cannabinoids to be used as adjuncts to thrombolytics. However, the *in vivo* effects of cannabinoids in situations of compromised cardiac function needs to be assessed as they could potentially cause excessive hypotension due to peripheral vasodilatation.

Chapter 1

General Introduction

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1.0 Introduction

Myocardial infarction, caused by coronary artery occlusion resulting in impaired blood supply (ischaemia), is a leading cause of morbidity and mortality in the western world. Although the incidence varies according to region, it is estimated that 147,000 men and 121,000 women of all ages suffer myocardial infarction each year in the UK (Petersen *et al.*, 2004).

1.1 Ischaemic injury

Myocardial cells gain their energy through glucose and fatty acid oxidation and oxygen-independent glycolysis (Theroux, 1999). During ischaemia the oxidative pathways are impaired which causes an increase in the rate of anaerobic glycolysis. Under these conditions, the hydrolysis of ATP derived from glucose metabolism becomes a significant source of hydrogen ions (Lopaschuk *et al.*, 1993). ATP hydrolysis is not the only mechanism to contribute to the acidosis seen during ischaemia. Protons can also be formed from carbon dioxide retention, NADH and hydrogen ion accumulation resulting from glutamate oxidation and from fatty acid recycling, however, it is debatable as to how much they contribute to hydrogen ion build up (Dennis *et al.*, 1991). The accumulation of hydrogen ions leads to the activation of the sodium/hydrogen exchange system which results in hydrogen ion removal from the cell in exchange for sodium resulting in cell swelling (Theroux, 1999). Activation of the sodium/potassium ATPase may also occur resulting in a decrease in intracellular sodium ion concentration, however, a low intracellular ATP level along with a low pH (both caused by the hydrolysis of ATP) are not favourable conditions for the functioning of the ATPase and therefore this exchange mechanism may not have a great

effect on lowering the intracellular sodium ion concentration (Van Emous *et al.*, 1998). Another system exchanging intracellular sodium for extracellular calcium which is stimulated by the abnormally high intracellular levels of sodium ions assists the sarcoplasmic reticulum in maintaining calcium ion homeostasis (Hasenfuss *et al.*, 1999; Tani & Neely, 1989). This further results in calcium overload, sarcolemmal bleb formation and rupture of the cell ultimately resulting in cell death (Theroux, 1999). Figure 1.1 summarises the consequence of acidosis induced by ischaemia and the mismatch between rates of glucose oxidation and glycolysis during reperfusion.

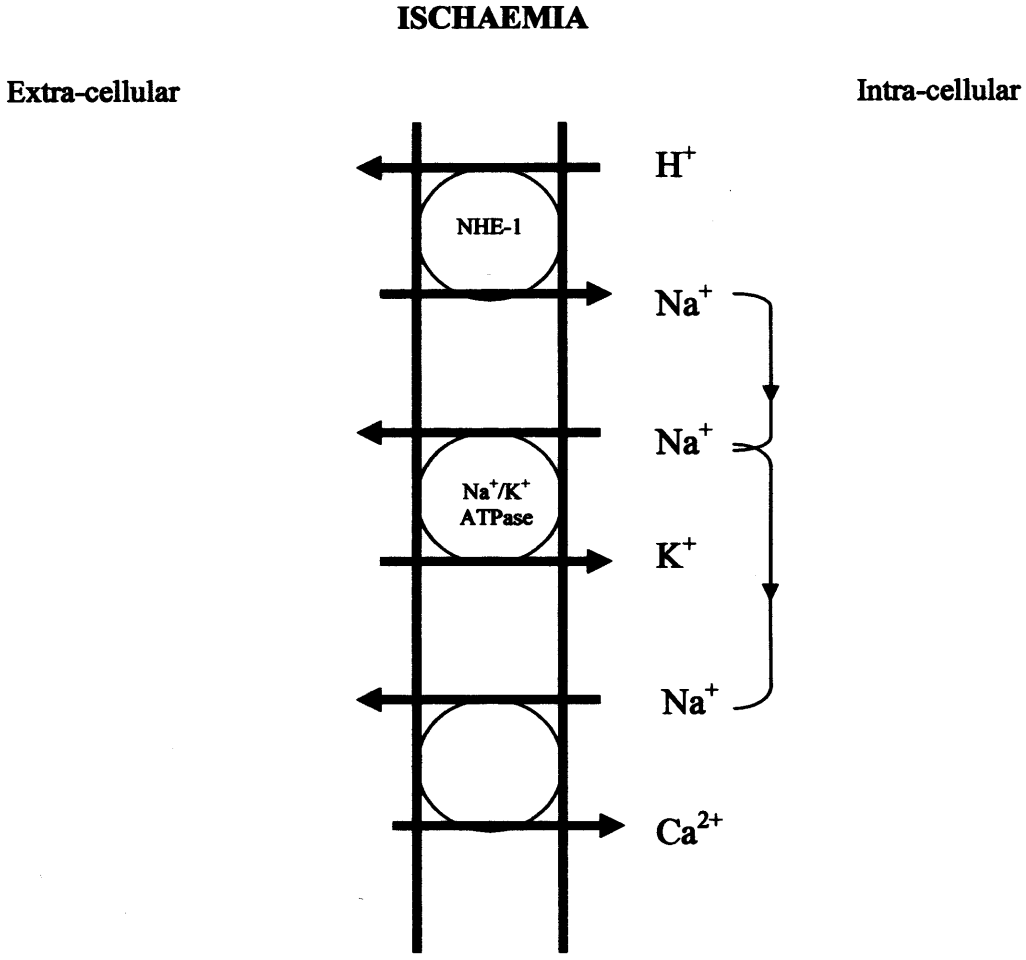

Figure 1.1 *Diagram showing the cascade of events leading to the increase in calcium caused by ischaemia. Glycolysis is increased while glucose oxidation is impaired resulting in an increase in hydrogen ions. The sodium/hydrogen exchange system (NHE-1) activated by high intracellular levels of hydrogen ions tries to compensate by exchanging hydrogen ions for sodium ions. The subsequent elevated intracellular levels of sodium ions activate the sodium/potassium ion (Na^+/K^+) ATPase but this is inhibited due to increased cellular ATP hydrolysis and a low pH. As the elevated intracellular levels of sodium ions are not adequately controlled by the Na^+/K^+ ATPase, sodium ions are exchanged for extracellular calcium ions via the sodium/calcium ion exchanger i.e. in reverse of its normoxic function. Extracellular calcium ion influx via this route contributes to the overall calcium ion overload observed in ischaemia-reperfusion injury.*

1.2 Reperfusion injury

Ischaemic myocardium will ultimately die if it is not reperfused. Reperfusion, restoration of flow after a period of ischaemia is currently the only means of salvaging ischaemic myocardium and limiting infarct development providing reperfusion occurs soon after occlusion (Yellon *et al.*, 1999). Although reperfusion is necessary, it is associated with additional injury processes, including contractile dysfunction (stunning), the development of arrhythmias, endothelial dysfunction and cell death collectively leading to a decrease in cardiac performance (Black, 2000; Park & Lucchesi, 1999; Piper *et al.*, 1998; Yellon & Baxter, 1999). Many mechanisms have been shown to contribute to reperfusion injury including oxygen free radical formation, calcium overload and neutrophil mediated myocardial and endothelium injury amongst others (Wang *et al.*, 2002). These mechanisms will be discussed individually in the following section.

The generation of oxygen free radicals (highly reactive species containing unpaired electrons) including the superoxide anion, hydroxyl radical and hydrogen peroxide, is a key process in the development of reperfusion injury. Reintroduction of oxygen at the onset of reperfusion results in the release of these free radicals and along with the ischaemia-induced decrease in antioxidant activity leaves the myocardium at risk of damage. Ultimately these oxygen free radicals react with cellular phospholipids and proteins resulting in the disruption of the membrane ultrastructure and dysfunction of cellular proteins (Wang *et al.*, 2002).

Calcium overload also causes problems during reperfusion, where intracellular calcium concentrations increase rapidly due to sodium influx into the cells (Buerke *et al.*, 1999). Calcium overload not only results from the activation of exchanger processes but also due to calcium gaining access through 'leaky' myocardial cells which have occurred as the result of morphological changes in the cellular structure i.e. cell swelling and disruption of the tissue lattice. A dysfunctional sarcoplasmic reticulum which has become damaged as a result of the generation of oxygen-derived free radicals and is unable to adequately sequester and store intracellular ionized calcium can also contribute to increased calcium levels (Park & Lucchesi, 1999).

Calcium overload has been found to contribute in several ways to reperfusion injury. At reoxygenation calcium induces excess myofilament activation and in addition causes an increase in mitochondrial calcium resulting in impairment of the mitochondria's ability to generate ATP therefore limiting metabolic recovery of the cell. Calcium activated proteases may also destroy intracellular structures (Wang *et al.*, 2002).

Although the inflammatory response is needed for healing and scar formation, it is also another process contributing to myocardial reperfusion injury. Neutrophils, a major cellular component of this process, not only gain access through extravasation into the endothelium but also by releasing proteases, pro-inflammatory mediators, oxygen metabolites and other cytotoxic substances which result in an increase in tissue injury (Frangogiannis *et al.*, 2002; Hansen, 1995; Jordan *et al.*, 1999).

Myocardial stunning is mechanical dysfunction, which persists after reperfusion, despite the absence of irreversible damage and in spite of restoration of the coronary flow. There are two hypotheses for this phenomenon, one being calcium overload and the other that oxygen-derived free radicals interact with cell membranes and essential proteins resulting in the alteration of membrane structures and dysfunction of various cellular proteins, leading to decreased function and reversible tissue injury (reviewed by Bolli & Marban, 1999). Recovery from stunning could be due to slow repair of oxidative damage and/or resynthesis of contractile proteins or reversal of intracellular Ca^{2+} overload. The two theories for stunning are not mutually exclusive and could both play a role in this process (reviewed by Bolli & Marban, 1999).

When nutrient and oxygen supply is restored to the cells as a result of reperfusion the cytosolic calcium ion concentration is still high. This can have a harmful effect in that an uncontrolled, excessive force may be generated resulting in hypercontraction which is sustained shortening and stiffening of the myocardium resulting from changes in intracellular calcium concentrations (Moens *et al.*, 2005). Inhibition of the contractile machinery during the first stage of energy recovery can prevent hypercontracture because it allows time for the cation concentrations to be corrected (Piper *et al.*, 1998). Correcting the cytosolic pH can also influence hypercontracture. The cytosolic pH is lowered during ischaemia due to anaerobic metabolism and the breakdown of ATP, producing an excess of hydrogen ions. The pH of the interstitial fluid is rapidly normalised upon reperfusion creating a gradient between that and the cytosol. This results in the activation of the sodium/hydrogen ion exchange system, which corrects the acidosis (Piper *et al.*, 1998). However acidosis has been shown to be a protective

feature and can inhibit the myofibrillary machinery therefore helping to prevent hypercontracture. This protection was demonstrated when ischaemic rabbit myocardium was reperfused with erythrocyte containing perfusate which was either acidotic (pH 6.6) or that contained 5-(N, N-dimethyl)-amiloride a sodium/hydrogen exchange system inhibitor. Reperfusion with either resulted in a marked decrease in cell death (Kaplan *et al.*, 1995; Piper *et al.*, 1998; Wang *et al.*, 2002).

Survivors of the initial ischaemia-reperfusion injury (the combined damage caused by ischaemia and reperfusion) are susceptible to remodelling (hypertrophy and change in shape) of the heart, which can lead to heart failure. Myocardial remodelling is a reversible process triggered by mechanical stretch. In the case of myocardial infarction, remodelling is asymmetric and related to infarct expansion, which is as a result of death and slippage of cardiomyocytes. Remodelling of the area as a result of expansion causes an increase in left ventricular mass, volume and shape (Swynghedauw, 1999). Figure 1.2 demonstrates the effect of remodelling over time.

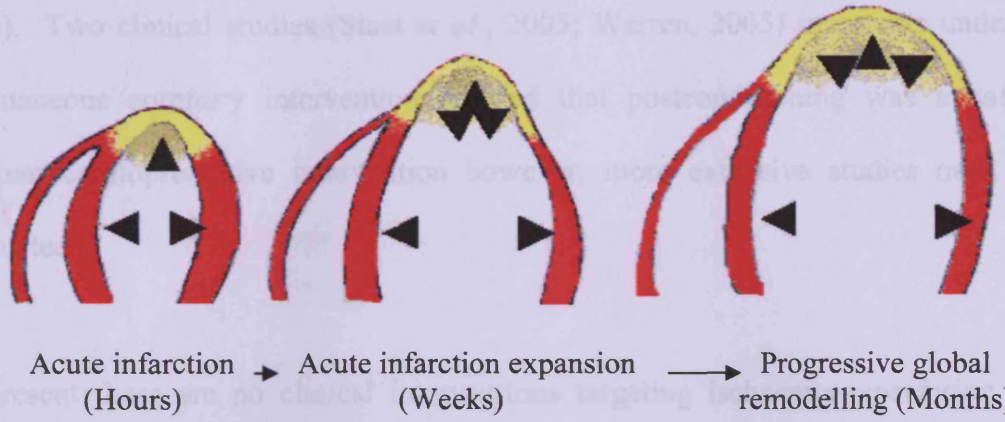

Figure 1.2 *Diagram of remodelling. Arrows indicate forces induced by intraventricular pressure. Arrows indicate intraventricular forces induced by contraction that cause stretching and thinning of the infracted area. Neurohumoral stimulation due to impaired contractile function stimulates hypertrophic growth of the whole heart resulting in cardiac hypertrophy.*

1.3 Interventions for cardiac damage

Besides early reperfusion, ischaemia-reperfusion injury can be reduced by a number of different interventions. Ischaemic preconditioning, first documented by Murry *et al.* (1986), is a powerful way of reducing the severity of ischaemia-reperfusion injury by subjecting the heart to a series of sub lethal ischaemic episodes. This phenomenon can be mimicked by numerous pharmacophores including adenosine (Mubagwa & Flameng, 2001) and opioids for example morphine and bradykinin B2-receptor agonists (Schultz & Gross, 2001). Unfortunately, the clinical application of this experimental finding is limited as the treatment needs to be applied before a coronary artery becomes occluded and most patients are asymptomatic before suffering their first heart attack (Moens *et al.*, 2005). Another phenomenon known as postconditioning which is defined as brief periods of reperfusion alternating with re-occlusion applied during the early stages of reperfusion also reduces infarct size (Penna *et al.*, 2006; Zhao & Vinten-Johansen,

2006). Two clinical studies (Staat *et al.*, 2005; Warren, 2005) in patents undergoing percutaneous coronary intervention showed that postconditioning was a safe and efficient cardioprotective intervention however, more extensive studies need to be conducted.

At present there are no clinical interventions targeting ischaemia-reperfusion injury apart from reperfusing the affected area soon after occlusion. Thrombolytics ('clot busting' drugs) are the group of drugs used as the standard treatment (Department of Health, 2000). As mentioned, there is experimental evidence to suggest that ischaemia-reperfusion injury can be limited through treatment. However, suggested treatments either have to be given before occlusion (e.g. preconditioning) or they can have a detrimental effect on those patients with compromised cardiac function (e.g. adenosine). Therefore there is a need to develop agents that can reduce ischaemia-reperfusion injury when provided as adjuncts to thrombolytics without significantly impairing contractile function.

Cannabinoids are a group of drugs, which have come to light recently as having influence on the cardiovascular system, with evidence suggesting a role in protection against ischaemia-reperfusion injury (Joyeux *et al.*, 2002; Lagneux & Lamontagne, 2001). Cannabinoids are the active constituents of marijuana (*cannabis sativa* plant), greater than 60 of which have been discovered. The most abundant cannabinoid is Δ^9-tetrahydrocannabinol, and it is this, which gives marijuana its psychotropic effects (Mechoulam *et al.*, 1998; Sugiura & Waku, 2002).

One of the most important developments in cannabinoid research was the discovery, in 1992, of the first endogenous cannabinoid agonist *N*-arachidonoylethanolamide (anandamide, Figure 1.3).

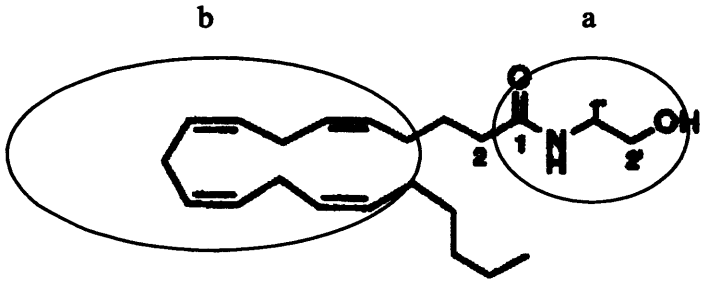

Figure 1.3 *Structure of anandamide. Anandamide consists of (a) a polar ethanolamide head group and (b) a hydrophobic arachidonoyl chain (Khanolkar & Makriyannis, 1999).*

The discovery of anandamide (Devane *et al.*, 1992) came at a similar time to that of the identification and cloning of the two cannabinoid receptors designated cannabinoid receptor 1 and 2 (CB_1 and CB_2 respectively; Matsuda *et al.*, 1990; Munro *et al.*, 1993).

1.4 Synthesis and metabolism of the endogenous cannabinoid anandamide

Two hypotheses have been suggested for anandamide biosynthesis. The first involves the activation of phospholipase A_2 and D respectively followed by the release of arachidonic acid and ethanolamine. Anandamide would be formed by anandamide synthase creating an amide bond. However, there is a flaw with this theory in that concentrations that would be required are in excess of those found physiologically (Devane & Axelrod, 1994; Kruszka & Gross, 1994). The second and more widely accepted hypothesis is one where acyltransferase changes the conformation of arachidonic acid by moving the position of the donor phospholipid to create an amide bond at the ethanolamide head group in the third position of the

phosphatidylethanamide. Activation of phospholipase D would then result in the formation of anandamide (Cadas *et al.*, 1997; Sugiura *et al.*, 1996a; Sugiura *et al.*, 1996b).

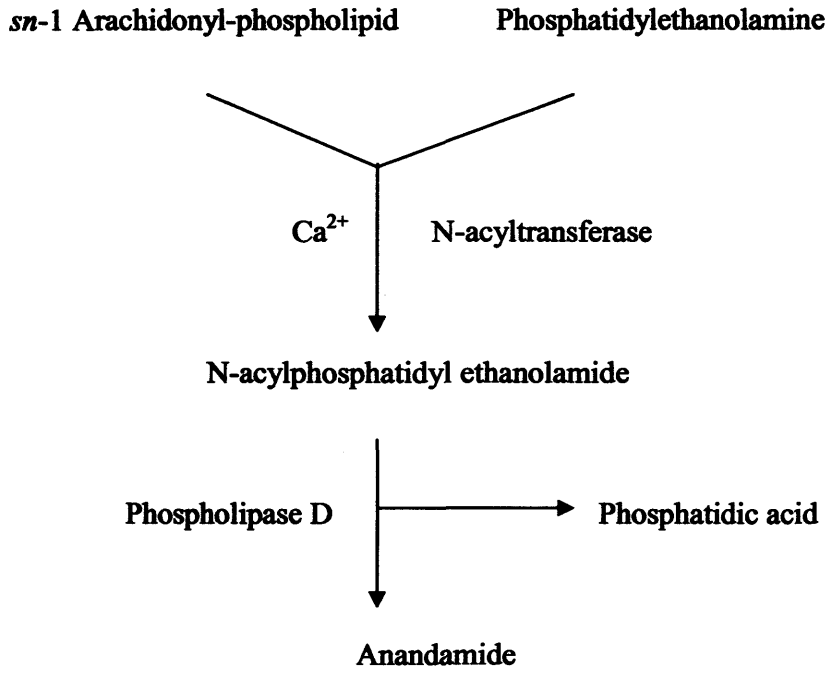

Figure 1.4 *Biosynthesis of anandamide from arachidonic acid. Increases in intracellular calcium cause a conformational change in arachidonic acid from the sn 1 position of an N-acylphospholipid to the amide group of the Phosphatidylethanolamine. Phospholipase D then cleaves a phosphodiester bond to create anandamide (Cadas et al., 1997; Felder & Glass, 1998).*

1.5 Uptake and degradation of endocannabinoids

Termination of the biological actions of anandamide appears to be a two step process, the first being transportation into the cell and secondly once inside the cell enzymatic hydrolysis by anandamide amidohydrolase, latterly known as fatty acid amide hydrolase. Fatty acid amide hydrolase is an intracellular, membrane-bound enzyme,

which is the primary enzyme responsible for the hydrolytic breakdown of anandamide into arachidonic acid and ethanolamine (Deutsch *et al.*, 2002; Khanolkar & Makriyannis, 1999). Fatty acid amide hydrolase has a similar distribution to CB_1 receptors in the central nervous system and it is shown to work in conjunction with other membrane proteins to facilitate anandamide transport by creating an inward concentration gradient as a result of anandamide metabolism (Deutsch *et al.*, 2001; Egertova *et al.*, 1998).

For anandamide to be metabolised by fatty acid amide hydrolase it must be transported into the cell. There are a number of theories by which anandamide uptake is thought to occur. The first is that anandamide is transported across the plasma membrane by a carrier protein (Hillard & Jarrahian, 2000). However, this theory is still controversial as this putative anandamide transporter has yet to be cloned. The second theory is that of simple diffusion where the fatty acid amide hydrolase-mediated cleavage of anandamide maintains a concentration gradient (Glaser *et al.*, 2003). The third hypothesis is that cellular components such as protein or lipid compartments can sequester anandamide (Hillard & Jarrahian, 2003). And the fourth theory is that anandamide can be internalised by a calveolae-mediated endocytic process (McFarland *et al.*, 2004). It is also possible that all these mechanisms exist.

Anandamide can also be metabolised by cyclooxygenase-2 (COX-2; but not COX-1), which is an inducible form of cyclooxygenase leading to the generation of a number of oxygenated products including PGE_2-ethanolamide (Yu *et al.*, 1997). However, the physiological significance of this pathway has yet to be discovered. Using

indomethacin (inhibitor of COX-2), can prevent biosynthesis into prostaglandin (Kozak & Marnett, 2002; Mechoulam *et al.*, 1998; Pertwee & Ross, 2002; Yu *et al.*, 1997).

Lipoxygenase mediated hydroxylations of anandamide analogues to those of arachidonic acid have also been reported (Burstein *et al.*, 2000). Anandamide has been shown to be metabolised to hydroperoxyeicosatetraenoyl ethanolamides which like the hydroperoxyderivatives of arachidonic acid act as TRPV1 (transient receptor potential vanilloid 1 cation channel, formally known as the vanilloid VR1 receptor) agonists (Craib *et al.*, 2001) and to hydroxyl-eicosatetraenoylethanolamides which have been shown to have similar affinity for both CB_1 and CB_2 receptors as anandamide itself (Edgemond *et al.*, 1998). However, the biological significance of lipoxygenase metabolism of cannabinoids is unclear.

Uptake inhibitors such as *N*-(4-hydroxyphenyl)arachidonylamide (AM404) have been developed (Beltramo *et al.*, 1997). This agent was discovered while studying the structure-activity relationships of anandamide, where it was found that by substituting the 2-hydroxyethyl group in anandamide with a 4-hydroxyphenyl group resulted in a competitive transport inhibitor i.e. it blocks the effects of both the anandamide transporter (Beltramo *et al.*, 1997; Khanolkar & Makriyannis, 1999) and fatty acid amide hydrolase (Jarrahian *et al.*, 2000). One problem with the use of this antagonist is that it can act as a TRPV1 agonist (Zygmunt *et al.*, 2000). However, AM404 can be used in conjunction with the TRPV1 antagonist, capsazepine, to exclude potentially confounding actions mediated by TRPV1 activation. Other transport inhibitors have since been developed including VDM 11 an anandamide membrane inhibitor which has

little or no activity at TRPV1 receptors and is also an inhibitor of fatty acid amide hydrolase (De Petrocellis *et al.*, 2000; Vandevoorde & Fowler, 2005), OMDM-2 an inhibitor of anandamide cellular uptake which does not cross react with fatty acid amide hydrolase (Ortar *et al.*, 2003) and UCM707 an uptake inhibitor which has no affinity for fatty acid amide hydrolase, TPRV1, CB_1 or CB_2 receptors (Lopez-Rodriguez *et al.*, 2003). As some of these inhibitors do not affect fatty acid amide hydrolase they provide further evidence that an anandamide transporter does exist.

Inhibitors have also been developed which prevent the hydrolysis of anandamide. These include palmitoylisopropylamide which is a fatty acid amide hydrolase inhibitor (Jonsson *et al.*, 2001) and methyl arachidonyl fluorophosphate which is an irreversible anandamide amidase inhibitor (Deutsch *et al.*, 1997). The effect of these inhibitors would be to decrease both the inward concentration gradient created by the hydrolysis of anandamide by fatty acid amide hydrolase and to decrease the efficiency of anandamide clearance (McFarland & Barker, 2004). These inhibitors would have therapeutic benefit over other ligands as they would act in a site and event specific manner.

1.6 Cannabinoid receptors

In the 1990's two cannabinoid receptors designated cannabinoid receptor 1 and 2 (CB_1 and CB_2 respectively) were identified and cloned (Matsuda *et al.*, 1990; Munro *et al.*, 1993). The locations of these receptors have been established through the use of quantitative autoradiography, in situ hybridisation and immunocytochemistry (Howlett *et al.*, 2002). CB_1 receptors were found to be widely distributed throughout mammalian

tissues with the greatest expression levels being in the nervous system. They were also found to a lesser extent in peripheral tissues including the heart, lungs and bone marrow. CB_2 receptors on the other hand were found to be expressed predominantly in immune tissues including the spleen, tonsils and lymph nodes. CB_2 mRNA has also been found to be expressed in several types of leukocytes including macrophages, monocytes, B lymphocytes and natural killer cells (Felder & Glass, 1998; Galiegue *et al.*, 1995; Howlett, 2002; Sugiura & Waku, 2002; Valk *et al.*, 1997).

The homology of cannabinoid receptors has also been investigated and revealed that the CB_2 receptor has only a 44% homology with the CB_1 receptor (Felder & Glass, 1998). However, the homology of CB_1 receptors across species (human, rat and mouse) is highly conserved with 97 to 99% amino acid identity. CB_2 receptors are more divergent with mouse CB_2 gene clones having only 82% overall identity with the human CB_2 receptor (Chakrabarti *et al.*, 1995; Shire *et al.*, 1996).

The use of agonist stimulation resulted in the discovery that responses mediated by cannabinoid receptors were due to their being coupled to G-proteins indicating they are members of the G-protein coupled receptor superfamily (Howlett *et al.*, 2002). Figure 1.5 summarises intracellular signalling pathways known to be coupled to cannabinoid 1 receptor activation.

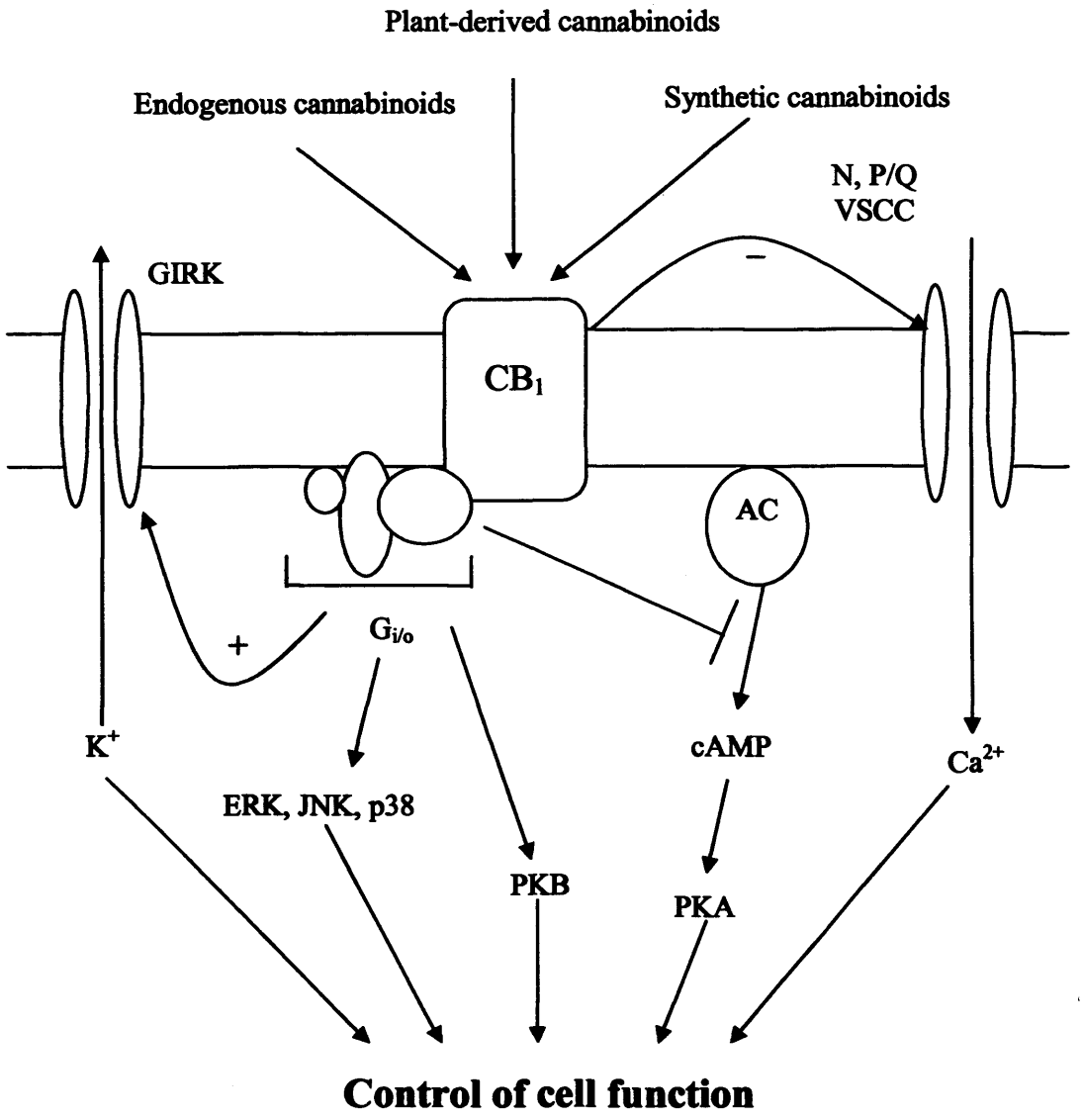

Figure 1.5 *Cannabinoids exert their effects by binding to specific $G_{i/o}$-protein-coupled plasma membrane receptors. The cannabinoid 1 receptor (CB_1) signals several different pathways including inhibition of adenylyl cyclase (AC), modulates ion channels (inhibits N- and P/Q type voltage sensitive calcium channels (VSCC), activates G-protein-activated inwardly rectifying potassium channels (GIRK), and activates mitogen- and stress-activated protein kinase cascades (ERK, JNK, p38), and protein kinase B (PKB), all resulting in control of cell function by cannabinoids. The cannabinoid 2 receptor couples to some but not all if the pathways shown (see review Howlett et al., 2002).*

In general, CB_1 and CB_2 receptors couple to $G_{i/o}$ receptors (Howlett *et al.*, 2002). However, under conditions where $G_{i/o}$ is inactivated by treatment with pertussis toxin, the CB_1 receptor has also been shown to interact with G_s proteins. Evidence that CB_1 receptors couple to G_s has been provided from investigations into the relationship between CB_1 receptors and D_2 (dopamine) receptors (Glass & Felder, 1997; Jarrahian *et al.*, 2004). In striatal neurones in primary culture activation of either CB_1 or D_2 receptors resulted in the inhibition of cyclic AMP accumulation whereas simultaneous activation of the receptors resulted in an augmentation of cyclic AMP accumulation. Pertussis toxin treatment (used to block G_i) of the neurones prevented the inhibition of adenylate cyclase by both the CB_1 and D_2 receptors and uncovered a CB_1 but not D_2 receptor-mediated stimulation of cyclic AMP. This response was blocked by SR141716A (CB_1 receptor antagonist now known as rimonobant) providing an alternative CB_1 receptor signalling pathway (Glass & Felder, 1997). A further study has demonstrated that CB_1 receptor activation inhibits cyclic AMP accumulation in the absence of D_2 receptors, whereas CB_1-stimulated cyclic AMP accumulation (through G_s) occurs in their presence. This G_s subunit switching was not prevented in the presence of pertussis toxin and occurred in the presence and absence of D_2 receptor activation (Jarrahian *et al.*, 2004).

The orphan G-protein-coupled receptor GRP55 has more recently come to light as a new cannabinoid receptor (Sawzdargo *et al.*, 1999). GRP55 has been shown to bind and be activated by endogenous (anandamide), natural (abnormal cannabidiol) and synthetic (CP55940) cannabinoid ligands. Therefore, GPR55 has been classed as a

novel cannabinoid receptor and a biological and pharmaceutical target of cannabinoid ligands (Drmota, 2004).

Other recent evidence supports the idea that cannabinoids can mediate responses through receptors other than CB_1 and CB_2 receptors. A novel endothelial cannabinoid receptor has been proposed on the basis of functional pharmacology but has yet to be cloned (Jarai *et al.*, 1999). Abnormal cannabidiol, a plant-derived cannabinoid, is a selective agonist (Jarai *et al.*, 1999; Offertaler *et al.*, 2003) and O-1918 is a selective antagonist (Offertaler *et al.*, 2003) of this receptor. Cannabinoid responses in $CB_1^{-/-}$ and $CB_2^{-/-}$ mice are thought to be due to activation of this potentially novel cannabinoid receptor (Jarai *et al.*, 1999; Offertaler *et al.*, 2003).

It has also been found that some cannabinoids are full agonists of TRPV1. Cannabinoids have been shown to produce similar inward currents to those of capsaicin and the responses to these cannabinoids are sensitive to the capsaicin antagonist, capsazepine (Bevan *et al.*, 1992; Di Marzo *et al.*, 1998; Smart & Jerman, 2000; Zygmunt *et al.*, 1999).

There are a number of reports that cannabinoid responses may be mediated by receptors other than those previously described. Of interest to the current work, anandamide responses in the heart have been shown to be sensitive to both SR141716A and SR144528 (CB_1 and CB_2 receptor antagonists respectively) but insensitive to agonists selective for CB_1 or CB_2 receptors, either alone or in combination (Ford *et al.*, 2002).

On the basis of these findings, the authors suggested that there may be a novel cannabinoid site of action in the heart.

In addition to direct interaction with extracellular receptors, cannabinoids can be taken up into cells and metabolised to products of the arachidonic acid cascade such as prostaglandins, thromboxane and leukotrienes (Belton & Fitzgerald, 2003; Ellis *et al.*, 1995; Kozak *et al.*, 2002). These pathways can be circumvented by the use of methanandamide a non-hydrolysable analogue of anandamide (Abadji *et al.*, 1994) and through the use of uptake inhibitors including OMDM-2 (Ortar *et al.*, 2003) and UCM707 (Lopez-Rodriguez *et al.*, 2003) and inhibitors of fatty acid amidase hydrolase including palmitoylisopropylamide (Jonsson *et al.*, 2001) and methyl arachidonyl fluorophosphates (Deutsch *et al.*, 1997).

1.7 Cannabinoid receptor ligands

The elucidation of the structure of anandamide has led to the development of several agonists with CB_1- or CB_2-selectivity. CB_1-selective agonists include *R*-(+)-methanandamide a non-hydrolysable analogue of anandamide (Abadji *et al.*, 1994), arachidonlycyclopropylamide and arachidonyl-2'-chloroethylamide more commonly known as ACPA and ACEA respectively (Hillard *et al.*, 1999) which are more selective than anandamide itself which is an agonist of both CB_1 and CB_2 receptors as well as acting as a full agonist of TRPV1 (Di Marzo *et al.*, 1998; Smart & Jerman, 2000; Zygmunt *et al.*, 1999). CB_2-selective agonists which include 3-(1'1'dimethylbutyl)-1-deoxy-Δ8-THC (Huffman *et al.*, 1999) more commonly known as JWH-133, HU-308 (Hanus *et al.*, 1999), L-759656 and L-759633 (Ross *et al.*, 1999) are derived from

modifications to the tetrahydrocannabinol molecule (Hanus *et al.*, 1999; Huffman *et al.*, 1999). Table 1.1 shows a comparison of the binding affinities of agonists for the classic cannabinoid receptors.

Table 1.1: *Comparison of the affinities (K_i) of cannabinoid ligands for the classic cannabinoid receptors.*

Cannabinoid Ligand	CB_1	CB_2	Reference
Agonists			
Anandamide	71.7nM	279nM	(Hillard *et al.*, 1999)
Methanandamide	20nM		(Abadji *et al.*, 1994)
ACEA	1.4nM	>2000nM	(Hillard *et al.*, 1999)
ACPA	2.2nM	715nM	(Hillard *et al.*, 1999)
JWH-133	677nM	3.4nM	(Huffman *et al.*, 1999)
HU-308	>10μM	22.7nM	(Hanus *et al.*, 1999)
AM281	12nM	4200nM	(Lan *et al.*, 1999)
L-759656	4888nM	11.8nM	(Ross *et al.*, 1999)
L-759633	1043nM	6.4nM	(Ross *et al.*, 1999)
Antagonists			
SR141716A	11.8nM	13200nM	(Felder *et al.*, 1998)
SR144528	437nM	0.6nM	(Rinaldi-Carmona *et al.*, 1998)
AM630	5152nM	31.2nM	(Ross *et al.*, 1999)
LY320135	141nM	14900nM	(Felder *et al.*, 1998)

Antagonists of both CB_1 and CB_2 receptors have also been developed. The first were SR141716A and SR144528, which are selective for CB_1 and CB_2 receptors, respectively (Rinaldi-Carmona *et al.*, 1995; Rinaldi-Carmona *et al.*, 1998). However, it is now apparent that these two antagonists can also act as inverse agonists (MacLennan *et al.*, 1998; Rinaldi-Carmona *et al.*, 1998) whereby they produce opposing effects to those observed with agonists. This could be the result of antagonism of endogenously released endocannabinoids, however some of these effects have been produced in the absence of any cannabinoid release (Pertwee, 2005). Analogues of SR141716A which include AM281 (Lan *et al.*, 1999) and AM251 (Gatley *et al.*, 1996) have also been shown to block the CB_1 receptor. Another CB_1 antagonist LY320135 has also been

developed (Felder *et al.*, 1998) but this compound has less affinity than SR141716A for CB_1 receptors. LY320135 has also been shown to bind to muscarinic and 5-HT_2 receptors in the low micromolar range (Felder & Glass, 1998) and it can behave as an inverse agonist at CB_1 receptors (Pertwee, 2005). 6-iodopravadoline (AM630; Hosohata *et al.*, 1997) is a selective CB_2 receptor antagonist and as with all the antagonists discussed it also acts as an inverse agonist (Ross *et al.*, 1999). This antagonist can also behave as a weak partial agonist at CB_1 receptors (Ross *et al.*, 1999). Table 1.1 shows a comparison of the binding affinities of antagonists for the classic cannabinoid receptors.

Responses can be pharmacologically distinguished with the use of selective agonists and antagonists and also with the use of transgenic mice which have been developed. These mice can be either CB_1 or CB_2 deficient or lack both of the receptors; therefore the responses produced can be definitively associated or discounted from cannabinoid receptor involvement.

1.8 Signal transduction

Cannabinoid receptors have been shown to couple to a number of signal transduction pathways. Table 1.2 lists a few of these pathways. Some of the pathways activated by cannabinoids are thought to be involved in influencing survival and apoptotic pathways. Activation of phosphatidylinositol 3-kinase and protein kinase B (Aikawa *et al.*, 2000) have been shown to mediate the inhibition of ischaemia/reperfusion-induced cardiac apoptosis (in the form of infarction) induced by insulin (Jonassen *et al.*, 2001). It is possible that cannabinoids limit apoptosis through this pathway as CB_1 receptors are

also coupled to phosphatidylinositol 3-kinase and protein kinase B activation (Gomez del Pulgar *et al.*, 2000). Supporting a role of cannabinoids in influencing both survival and pro-apoptotic pathways is the finding that the endocannabinoid, anandamide has been shown to be both anti- and pro-apoptotic (Maccarrone *et al.*, 2000b). Anandamide was shown to inhibit apoptosis through a receptor sensitive to both SR141716A and SR144528 and induce apoptosis through TRPV1 activation in human neuroblastoma and lymphoma cells (Maccarrone *et al.*, 2000b).

Table 1.2: *Cannabinoid receptor-coupled signalling transduction pathways*

Receptor	Signalling target	Effect	References
CB_1	Adenylate Cyclase	Inhibition	(Felder *et al.*, 1993) (Wade *et al.*, 2004)
	p38 MAPK	Stimulation	(Derkinderen *et al.*, 2001)
	p42/44 MAPK	Stimulation	(Bouaboula *et al.*, 1995) (Derkinderen *et al.*, 2003)
	PI3/protein kinase B	Stimulation	(Sanchez *et al.*, 1998) (Galve-Roperh *et al.*, 2002)
	Voltage-operated calcium channels		
	N-type	Inhibition	(Pan *et al.*, 1996) (Wilson *et al.*, 2001)
	P/Q type	Inhibition	(Twitchell *et al.*, 1997) (Hampson *et al.*, 1998)
	L-type	Inhibition	(Gebremedhin *et al.*, 1999) (Straiker *et al.*, 1999)
	Potassium channels		
	G-protein-coupled inwardly rectifying potassium channel	Stimulation	(McAllister *et al.*, 1999) (Robbe *et al.*, 2001)
	I_A	Stimulation	(Hampson *et al.*, 1995) (Mu *et al.*, 2000)
	I_D	Inhibition	(Mu *et al.*, 1999)
	I_M	Inhibition	(Schweitzer, 2000)
CB_2	Adenylate Cyclase	Inhibition	(Bayewitch *et al.*, 1995)
	P42/44 MAPK	Stimulation	(Bouaboula *et al.*, 1996)

1.9 Biological activity of cannabinoids

As previously stated anandamide acts as a full agonist at TRPV1 (Zygmunt *et al.*, 1999). Activation of these channels results in a vasodilator response and the induction of apoptosis in neuronal and immune cells (Jeon *et al.*, 1996; Maccarrone *et al.*, 2000a; Zygmunt *et al.*, 1999).

Cannabinoids have been shown to be able to inhibit macrophage activity (Burnette-Curley *et al.*, 1993; Tang *et al.*, 1993). Macrophages play an important part in local host defence mechanisms by recognising, phagocytosing, and destroying the foreign agent. Anandamide and 2-arachidonyl-glyceryl have been shown to be synthesised by macrophages (Di Marzo *et al.*, 1999; Di Marzo *et al.*, 1996; Kozak *et al.*, 2000; Pestonjamasp & Burstein, 1998; Varga *et al.*, 1998) which have also been shown to express both CB_2 and to a lesser extent CB_1 receptors (Bouaboula *et al.*, 1993; Galiegue *et al.*, 1995). Although cannabinoids are well established immune modulators their physiological relevance is still unclear.

1.10 Cardiovascular effects of cannabinoids

The most prominent component of the cardiovascular action of exogenous cannabinoid administration is prolonged hypotension (a triphasic response) and bradycardia. When a bolus of anandamide was injected into rats it resulted in a transient, vagally mediated bradycardia associated with a fall in blood pressure (phase I) which was followed by a brief pressor response (phase II) and a more prolonged decrease in blood pressure (phase III; (Varga *et al.*, 1995). Figure 1.6 shows a trace of this triphasic effect.

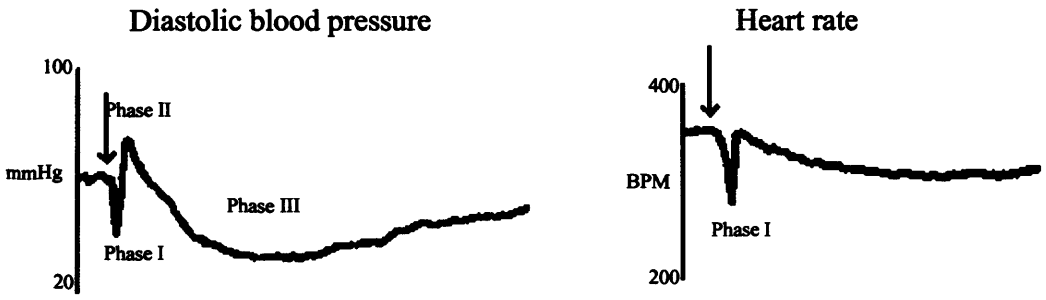

Figure 1.6 *Typical trace showing effect in rats of an intravenous injection of anandamide (1mg/kg) on diastolic blood pressure and heart rate (beats/minute, BPM). Arrows indicate drug application (Malinowska et al., 2001).*

Investigation of this triphasic effect found that phase I was due to activation of the TRPV1 as the selective TRPV1 antagonist capsazepine (Bevan *et al.*, 1992) reduced the response to anandamide and methanandamide (a stable analogue of anandamide). Phase III was found to be a result of CB_1 receptor activation because SR141716A abolished the effect caused by both anandamide and methanandamide (Malinowska *et al.*, 2001). Both the effects in phase III were absent in CB_1 receptor knockout mice further confirming that CB_1 activation caused this effect (Jarai *et al.*, 1999). The mechanism behind the brief vasopressor response (phase II) is unknown although this response was not inhibited by either capsazepine or SR141716A therefore there is a lack of both TRPV1 and CB_1 involvement (Lake *et al.*, 1997; Malinowska *et al.*, 2001).

In isolated rat hearts, Ford *et al.* (2002) showed that anandamide and methanandamide could significantly reduce coronary perfusion pressure, which is indicative of coronary vasodilatation. Left ventricular developed pressure, a measure of workload/muscle function was also reduced. Responses to anandamide were sensitive to both SR141716A and SR144528 but could not be explained by synergistic activation of both

CB_1 and CB_2 receptors. Therefore it was concluded that in the rat heart responses to anandamide were mediated via one or more novel sites.

In the 1970's *N*-acylethanolamine (anandamide) was shown to accumulate in infarcted dog hearts (Epps *et al.*, 1979) demonstrating that cannabinoids accumulate under pathological conditions. This could be because cannabinoids are retaliatory metabolites which are released in response to injury to limit tissue damage. It is therefore not surprising that cannabinoids have been found to be cardioprotective with endogenous cannabinoids being shown to mediate cardiac preconditioning (see 2.2.4a-c) induced by lipopolysaccharide, where SR144526 (but not SR141716A) was shown to abolish the protective effect of pre-treatment with lipopolysaccharide providing evidence of a CB_2 mediated effect (Lagneux & Lamontagne, 2001).

Endocannabinoids are also thought to directly protect against ischaemia-reperfusion injury with *sn*-2 arachidonyoylglycerol (another endogenous cannabinoid) and palmitoylethanolamide but not anandamide improving myocardial recovery, decreasing levels of biochemical markers (creatine kinase, lactate dehydrogenase) associated with ischaemic injury and reducing infarct size (Lepicier *et al.*, 2003). This protective mechanism was attributed to the activation of the CB_2 receptor as the response to *sn*-2 arachidonyoylglycerol was blocked by SR144528 and only partially blocked by SR141716A. It was also concluded that this protection involved the activation of signal transduction factors including p38, ERK1/2 and protein kinase C activation.

The cannabinoids anandamide and 2-arachidonoyl glycerol have been shown to be generated in monocytes and platelets following a myocardial infarction. CB_1 receptor inhibition was shown to prevent hypotension but decreased the 2 hour mortality rate from 70% in control animals to 36% in those treated with SR141716A (Wagner *et al.*, 2001a). This means that cannabinoids are important at the time of infarction because of their vasodilatory effect and ability to alter cytokine production (Klein *et al.*, 2000) and during the recovery process where vasodilation caused by the cannabinoids helps to maintain tissue perfusion when there is decreased cardiac output and compensatory sympathetic vasoconstriction (Wagner *et al.*, 2001a).

1.11 Current applications of cannabinoid use

Marijuana has been used for thousands of years as a therapeutic and recreational drug. Its therapeutic potential was documented as early as the 4th century BC when a Chinese emperor was using Marijuana to treat malaria, constipation and rheumatic pains (Felder & Glass, 1998).

In Western Europe, cannabis is the most used illicit drug. However, no deaths have been attributed to its use either recreationally or therapeutically probably because acute toxicity of cannabinoids is very low. Cannabis use does have adverse effects including sedation, psychological effects (anxiety, panic), physical effects (muscle weakness, tachycardia and hypotension) and impairment of psychomotor and cognitive performance (Kumar *et al.*, 2001).

Recently cannabinoid use in adolescence has been linked to an increased risk of psychosis. A number of studies have been conducted which have found that the use of cannabis is associated with an increased risk of psychosis or psychotic episodes (Andreasson *et al.*, 1987; Arseneault *et al.*, 2002; Caspi *et al.*, 2005; Fergusson *et al.*, 2005; Henquet *et al.*, 2005; van Os *et al.*, 2002). These studies not only compared cannabis users to non-cannabis users but some also looked at the effects of increasing cannabis use which again resulted in an associated increased risk of psychosis (Andreasson *et al.*, 1987; Fergusson *et al.*, 2005; van Os *et al.*, 2002).

Although there are adverse effects related to the use of cannabis, cannabis also has therapeutic applications. These are due to receptors (CB_1 predominantly) being located in the central and peripheral nervous system and include treatment of spastic disorders including multiple sclerosis. There is much anecdotal evidence from multiple sclerosis sufferers that cannabis relieves their symptoms such as spasticity, tremors and pain. One study used a chronic relapsing allergic encephalomyelitis mouse model to investigate the effects of cannabinoids on an experimental model of multiple sclerosis (Baker *et al.*, 2000). It was found that the cannabinoid agonists, WIN 55,212, tetrahydrocannabinol, methanandamide and JWH-133 tonically controlled both spasticity and tremor and that CB_1 and CB_2 cannabinoid antagonists exacerbated these effects. It has been suggested that this exacerbation is due to the inhibition of a tonically active, endogenous control mechanism (Baker *et al.*, 2000).

Clinical trials of cannabinoid based medicines for the treatment of multiple sclerosis have been conducted however; the results were inconclusive with participants reporting

feeling improvements that could not be confirmed by the study physicians. Therefore based on the studies to date, it is the opinion of the National Multiple Sclerosis Society's Medical Advisory Board, that there is currently insufficient data to recommend marijuana or its derivatives as a treatment for multiple sclerosis. However, in Canada a cannabis-derived drug, Sativex, is licensed for the treatment of pain associated with multiple sclerosis. The approval of the license was based on a four-week clinical trial conducted in 66 people with multiple sclerosis (Rog *et al.*, 2005). However, Sativex has not been licensed in the United States and GW Pharmaceuticals who developed this drug are currently conducting a clinical trial to evaluate it as a treatment for spasticity.

Cannabinoids have been indicated for anti-emetic use. Anti-emetic drugs are administered before and after chemotherapy when nausea and vomiting are frequent. At present dronabinol (tetrahydrocannabinol capsules) or the synthetic analogue nabilone (tetrahydrocannabinol in sesame oil) are approved for this use. Although the common side-effect (drowsiness) is problematic (Guzman, 2003; Kumar *et al.*, 2001).

Cannabinoids have been shown to have analgesic effects but in a review of controlled trials by (Campbell *et al.*, 2001), it was concluded that a single dose of cannabinoid was no more effective than codeine in treating nociceptive pain and that there was not enough evidence to suggest introducing cannabinoids into clinical practice for the treatment of pain. This was reinforced by an investigation into the use of dronabinol, in which patients with chronic refractory neuropathic pain did not have any benefit from the therapy (Attal *et al.*, 2004). However, it was suggested that the route of administration of cannabinoids may be a major factor in any future therapies as it is

known that tetrahydrocannabinol undergoes variable absorption in the gut (Kumar *et al.*, 2001) therefore getting the dosage for the individual right may be difficult.

It is the effects, which cannabinoids elicit on the cardiovascular system, in particular protecting the heart from injury, which has prompted this study. The aims of the study are to characterise cannabinoid-induced cardioprotection and investigate the mechanisms by which this protection is mediated. It is hoped that these studies will shed light on whether cannabinoids might be effectively used as adjuncts to thrombolytics to reduce ischaemia-reperfusion injury associated with heart attacks.

Chapter 2

General Methods

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2.0 General Methods

2.1 Animals and animal husbandry

Male Wistar rats weighing 250-300g at purchase were group housed, in numbers up to five, in standard plastic polypropylene rat cages (35cm x 55cm approximately). The ambient room temperature was 19-23°C, in accordance with Home Office Code of Practice regulations, and the rats were maintained under a 12:12 h, light: dark photoperiod (8am - 8pm light). The rats were allowed free access to a standard rodent maintenance diet and drinking water. Animal care and procedures were according to the Animals (Scientific Procedure) Act, UK, 1986.

2.2 Preparation of isolated right atria

Male Wistar rats (300-350g) were killed with an overdose of sodium pentobarbital (i.p., 0.2g/kg, Sagatal; Rhone Merieux, Harlow, UK). The skin was incised by a longitudinal cut from the middle of the abdomen to the throat. The abdomen was then opened up to the diaphragm. The diaphragm was cut away from the ribs. The thorax was cut open on the left and right sides of the rib cage, starting at the diaphragm and proceeding to the first rib. The complete anterior thoracic wall was then removed to expose the heart.

Right atria were removed, complete with the sino-atrial node, using threads tied to the superior vena cava, which attached the tissue to an isometric force transducer. Two threads, one attached to the inferior vena cava and the other attached to the atrioventricular junction, were used to secure the preparation to a stainless steel support. A resting tension of approximately 1g was applied. Right atrial preparations were allowed to beat spontaneously.

The tissue was suspended in 20ml tissue baths containing Krebs bicarbonate solution maintained at 37 ± 0.5°C by a Grant W6 circulator (Cambridge, UK) and continuously bubbled with a gas mixture of 95% O_2/5% CO_2. The Krebs bicarbonate solution was made up with deionised water and had the following composition (mM): NaCl (118.4); KCl (4.7); $CaCl_2.2H_2O$ (2.5); $MgSO_4.7\ H_2O$ (1.2); $NaHCO_3$ (24.9); $KH_2PO_4.2H_2O$ (1.2); glucose (11.6). All salts were obtained from Fisher Scientific (Leicestershire, UK).

The tensions developed by the right atrial preparations were measured by Dynamometer UF1 Isometric Force Transducers linked to a Grass pre-amplifier model 7P17 Driver Amplifier (Astro-Med Inc, West Warwick, USA) connected to a PC using an ADInstruments PowerLab 4/20 analogue digital converter (Oxfordshire, UK). Tension development and heart rate were continuously displayed and recorded on a PC.

An equilibration period of approximately 30 minutes was allowed before any experimental protocol commenced. During this period the tissue was washed every 10 minutes with fresh Krebs bicarbonate solution.

2.3 Langendorff buffered-perfused rat hearts

Male Wistar rats (300-350g) were killed with an overdose of sodium pentobarbitone (i.p, 0.2g/kg, Sagatal; Rhone Merieux, Harlow, UK) and heparinized (100 U kg-1 i.p, CP Pharmaceuticals, Wrexham, UK) to prevent the formation of blood clots in the heart. The abdomen was opened up to the diaphragm by making a longitudinal cut from the middle of the abdomen to the throat. The diaphragm was then cut from the ribs and

the thorax was cut open on the left and right sides of the rib cage, starting at the diaphragm and proceeding to the first rib. The ribcage was then removed to expose the heart. The heart and lungs were then removed together and immersed in a beaker of ice-cooled Krebs bicarbonate solution.

Hearts were cannulated within 90 seconds of excision and perfused at a constant pressure of 80mmHg (Langendorff perfusion, Figure 2.1) with perfusate consisting of a modified Krebs bicarbonate solution (NaCl 118 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, 2.5 mM Ca^{2+}, 11 mM glucose, 100 mU/l insulin). Care was taken not to insert the cannula too far into the aorta, which would occlude the coronary ostia or damage the aortic value. To prevent air bubbles from accumulating in the cannula, the perfusion fluid was allowed to flow through the cannula prior to contact with the aorta (leaving a drop on the tip of the cannula) and through use of a bubble trap at the top of the cannula. The heart was temporarily held in place by an aorta clip while the lungs and other connective tissues were removed from the heart. The aorta was then securely attached to the glass cannula using a cotton thread. A small incision was then made in the pulmonary artery to facilitate drainage of coronary perfusate and remnants of tissue (trachea, lung or connective tissue) were removed. The left atrium was removed to accommodate the placement of a pressurized (5-15 mmHg) balloon inserted into the left ventricle, connected to a pressure transducer (Ohmeda, model P23XL-1), which was further connected to a Grass pre-amplifier model 7P1G (Astro-Med Inc, West Warwick, USA). Coronary flow was measured by ultrasonic flow probes (Transonic H4X) connected to a Transonic Systems Inc small animal blood flow meter model T206 (Transonic Systems Inc, Ithaca, New York, USA). Electrical activity was continuously

recorded by means of two stainless steel needles inserted into the ventricular apex and base, connected to an electrocardiograph amplifier (Grass EKG Tachograph pre-amplifier model 7P4F, Astro-Med Inc, West Warwick, USA). Where electrical pacing was applied, hearts were electrically stimulated at 5Hz with square wave pulses of 5ms duration at a voltage of threshold + 10% via bipolar platinum electrodes placed on the surface of the heart. Depending on the protocol, some hearts were allowed to beat spontaneously as indicated in the relevant sections.

Hearts were immersed in 100ml organ baths containing perfusate maintained at 37°C ± 0.5°C by a Grant W6 circulator (Cambridge, UK). The perfusate was continuously bubbled with a gas mixture of 95% O_2/5% CO_2. Salts were obtained from Fisher Scientific (Leicestershire, UK) and insulin (stock concentration of 1000u/L) was obtained from LEO Pharma (Ballerup, Denmark).

All parameters (left ventricular developed pressure, heart rate and coronary flow) were continuously recorded using a PowerLab 800 (ADInstruments, Oxfordshire, UK) and digitally stored on a PC. Rate pressure product was calculated as the left ventricular developed pressure (mmHg) x heart rate (bpm).

Two protocols for the constant pressure Langendorff perfusion system were used in this study. Firstly, a no-flow global model of ischaemia (4.2) was used. In this model global ischaemia is induced by clamping the inflow line and the heart reperfused by releasing the clamp. The second model is that of regional ischaemia (5.2). In this

model, an area of the heart is made ischaemic by tying a snare around the left anterior descending coronary artery. The heart is reperfused by releasing the snare.

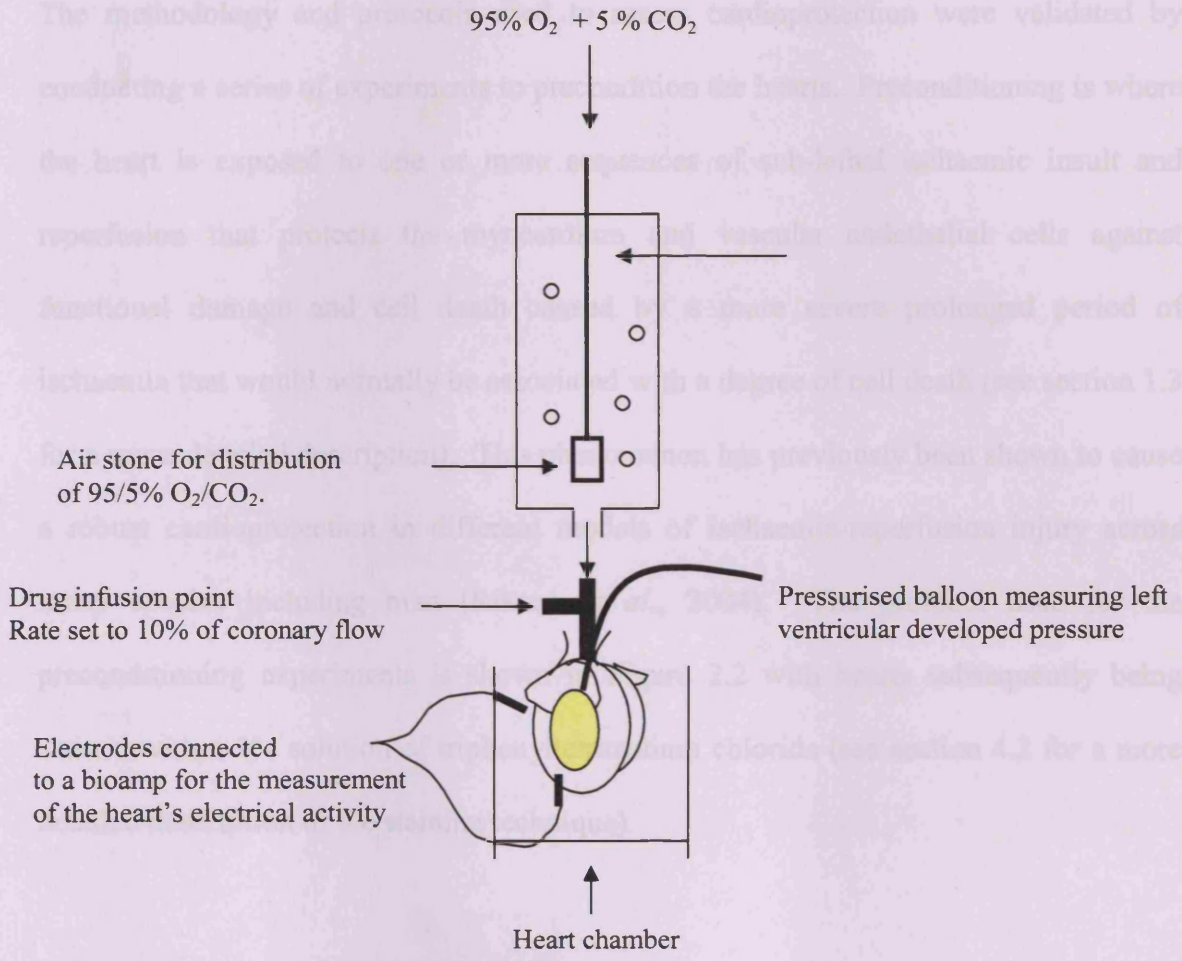

Figure 2.1 *The Langendorff constant pressure heart perfusion system. All parts of the apparatus are water-jacketed with warm circulating water to maintain the temperature of the perfusate and the heart at 37°C.*

2.4 Assessment of perfusion methodology for identifying cardioprotective responses

2.4a Protocol

The methodology and protocols used to assess cardioprotection were validated by conducting a series of experiments to precondition the hearts. Preconditioning is where the heart is exposed to one or more sequences of sub-lethal ischaemic insult and reperfusion that protects the myocardium and vascular endothelial cells against functional damage and cell death caused by a more severe prolonged period of ischaemia that would normally be associated with a degree of cell death (see section 1.3 for a more detailed description). This phenomenon has previously been shown to cause a robust cardioprotection in different models of ischaemia-reperfusion injury across many species including man (Riksen *et al.*, 2004). The protocol used for the preconditioning experiments is shown in Figure 2.2 with hearts subsequently being stained with a 1% solution of triphenyltetrazolium chloride (see section 4.2 for a more detailed description of the staining technique).

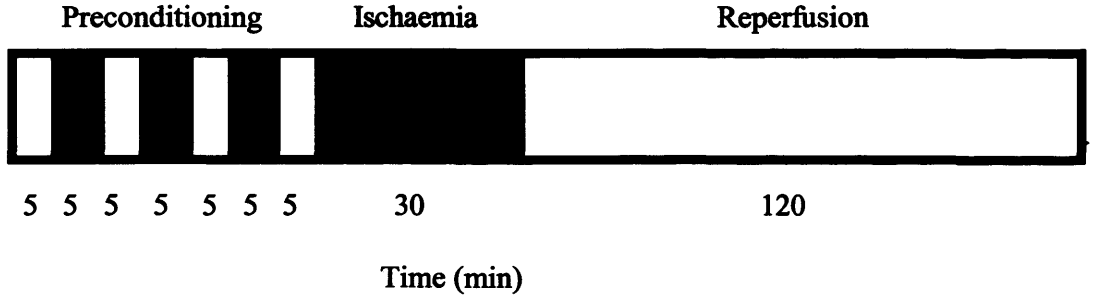

Figure 2.2 *Preconditioning protocol. There are three brief intermittent periods of ischaemia (filled boxes) and reperfusion (open boxes) before a prolonged period of ischaemia.*

2.4b Results

The results from the preconditioning experiments showed that preconditioning significantly improved recovery of left ventricular developed pressure but not coronary flow above that of the control group (Figure 2.3a and b). Preconditioning also significantly reduced infarct size from 28 ± 3% to 16 ± 6% (of the whole heart, Figure 2.3c). The effect on heart rate was not assessed because hearts were paced to 300 beats per minute.

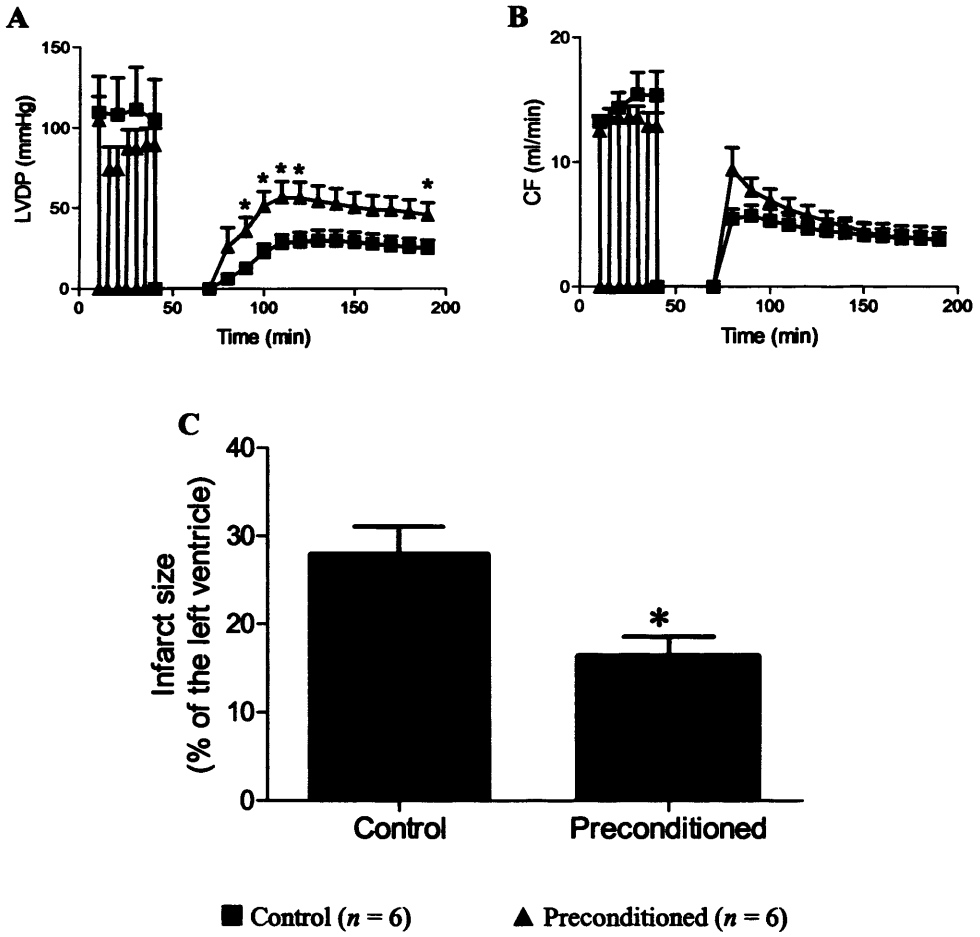

Figure 2.3 *Assessment of the effect of preconditioning on rat isolated Langendorff-perfused hearts subjected to 30 min global, no-flow ischaemia and 2 hour reperfusion. A. Effect of preconditioning on left ventricular developed pressure (LVDP). B. Effect of preconditioning on recovery of coronary flow (CF). C. Effect of preconditioning on infarct size. * represents P<0.05 using unpaired Student's t-test.*

2.4c Discussion

The results seen in the validation study show that the methodology and protocols used in this report are justified as preconditioning was shown to protect the heart. Although the cardioprotection seen was significant it was not to the same extent as previous studies which have seen a decrease from an infarct of 29.4% of the area at risk in control hearts to 7.3% in preconditioned hearts, however, dogs were used in those experiments whereas rats where used here and hearts were subjected to four 5 minute episodes of ischaemia whereas in the current study, hearts were subjected to three 5 minute episodes (Murry *et al.*, 1986). Thus it is possible that differences in animals and methodologies may account for the disparity in infarct size reduction observed.

2.5 Global versus regional ischaemia

The main difference between the global and regional ischaemia models is the damage which is caused. In regional ischemia, the tissue is damaged by the lack of oxygen, by mechanical stress, and by the consequences of a low-flow condition caused by collateral flow whereas in global ischemia the lack of oxygen is the only cause of cellular injury (Schaper, 1980).

There are few clinical situations where hearts are subject to global no-flow ischaemia. Global no-flow models of ischaemia are mainly limited to cardiac transplantation and cardiac surgery where the aorta is cross-clamped, whereas regional ischaemia models try to replicate the most common clinical manifestation of cardiac ischaemia, which occurs during myocardial infarction. In this case an occlusion occurs in a coronary

blood vessel resulting in disruption of blood supply to an area of the myocardium and reperfusion is instigated through the use of thrombolytic drugs or primary angioplasty. The regional ischaemia model is the closest to the most common clinical situation (myocardial infarction). However, this model does have its own drawbacks in that the clinical manifestation of regional ischaemia is associated with underlying pathophysiologies such as atherosclerosis, which are not present in the experimental method. In addition, reperfusion would be considerably slower in the clinical situation than in the mechanical model due to the time it would take for the clot to be broken down by the thrombolytics, although a faster reperfusion might be obtained with primary angioplasty. Although the extent of damage and initial condition of the hearts may differ between the experimental model and the clinical situation, the mechanisms are likely to be similar. Thus, treatments effective at reducing ischaemia-reperfusion damage experimentally would be likely to do the same in the clinical situation.

The global model gives a maximal area at risk (*i.e.* the whole heart) therefore it is a good model to use initially as potential changes in infarct size will be easier to detect. It could also be said that the global model could represent the area at risk of a larger animal *i.e.* that of a human and would therefore be more relevant to the clinical situation. However, there is the problem in that it is difficult to administer drugs only at reperfusion due to a lack of certainty that coronary flow is uniform across the whole heart. This means that the best time for drug administration is before ischaemia at which time it is impossible to distinguish between a preconditioning type of response and a direct cardioprotective response. It has previously been suggested that any stress to a

heart for example repeated episodes of angina could induce a preconditioning-like state (Murry *et al.*, 1986).

Many different models have been proposed to evaluate ischaemia-reperfusion injury with their overall aim to mimic the clinical situation. However, as you move away from the study of human tissue the model becomes less relevant to the human condition (Figure 2.5 demonstrates this issue).

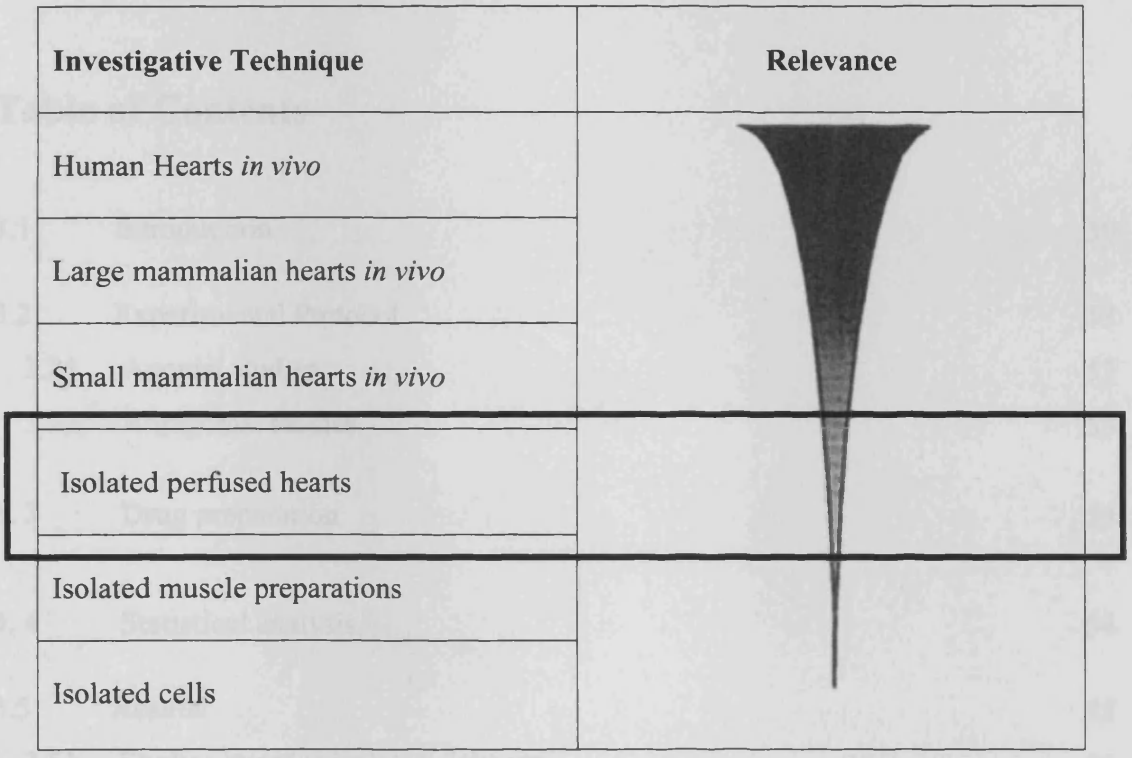

Figure 2.5 *Diagram showing the clinical relevance of different investigative techniques. The Langendorff perfused heart method has been highlighted to demonstrate its bearing (adapted from (Hearse & Sutherland, 2000).*

Chapter 3

Pharmacological evaluation of cannabinoid responses in isolated tissue preparations taken from rat hearts.

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3.1 Introduction

Cannabinoids, including the endogenous cannabinoid anandamide, have been shown to elicit numerous effects on the heart both *in vitro* and *in vivo*. However, the results from these studies have been inconsistent when the effects of cannabinoids on heart rate have been studied.

Using isolated perfused hearts, it was found that plant derived cannabinoids such as Δ^9-tetrahydrocannabinol and cannabinol cause tachycardia whereas cannabidiol caused bradycardia (Smiley *et al.*, 1976). Few studies have determined the direct effects of cannabinoids on isolated tissue preparations of the heart.

Other studies have investigated the effect of cannabinoids on force of contraction in electrically stimulated atrial muscle where it has been shown that anandamide, methanandamide and HU-210 all cause negative inotropy (Bonz *et al.*, 2003). The anandamide response was shown to be due to CB_1 receptor activation because AM251, a CB_1 receptor selective antagonist, inhibited the negative inotropic response (Bonz *et al.*, 2003).

The rate of spontaneous contraction in isolated right atria is derived from the activity of the sino-atrial node which is the pacemaker of the heart. This spontaneous heart rate is governed by the rate of depolarisation of the pacemaker cells (Satoh, 2003). Anandamide has been reported to have no effect on neuronally enhanced spontaneous rate (Lay *et al.*, 2000). However, in that study, the effect of cannabinoids on spontaneous rate was not determined.

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Other studies have investigated the effect of cannabinoids on force of contraction in electrically stimulated atrial muscle where it has been shown that anandamide, methanandamide and HU-210 all cause negative inotropy (Bonz *et al.*, 2003). The anandamide response was shown to be due to CB_1 receptor activation because AM251, a CB_1 receptor selective antagonist, inhibited the negative inotropic response (Bonz *et al.*, 2003).

The rate of spontaneous contraction in isolated right atria is derived from the activity of the sino-atrial node which is the pacemaker of the heart. This spontaneous heart rate is governed by the rate of depolarisation of the pacemaker cells (Satoh, 2003). Anandamide has been reported to have no effect on neuronally enhanced spontaneous rate (Lay *et al.*, 2000). However, in that study, the effect of cannabinoids on spontaneous rate was not determined.

In vivo, cannabinoids induce a triphasic hypotensive profile (Lake *et al.*, 1997a; Varga *et al.*, 1995). Initially there is a brief decrease in heart rate and blood pressure followed by a short-term vasopressor response followed by a prolonged decrease in heart rate. The principle fall in heart rate is related to the activation of TRPV1 which are primarily located on sensory neurones (Malinowska *et al.*, 2001), and where anandamide is a full agonist (Smart *et al.*, 2000). The later hypotensive response is sensitive to SR141716A and therefore has been ascribed to activation of CB_1 receptors (Malinowska *et al.*, 2001).

It is unclear whether the cannabinoid induced effects seen on heart rate are due to the influence of either the central or peripheral nervous system. By using rat spontaneously beating isolated right atria, the direct effects of cannabinoids on sino-atrial function can be studied without the involvement of potentially confounding responses mediated at the level of the central and peripheral nervous system. In addition, the potential role of interaction with β-adrenoceptors was investigated by studying responses to cannabinoids in rat spontaneously beating isolated right atria treated with isoprenaline.

Isoprenaline stimulates β-adrenoceptors causing the activation of adenylate cyclase leading to increased intracellular levels of the secondary messenger, cyclic AMP, which mediates an increase in heart rate (Whalen & Lewis, 1999). Cannabinoid-induced inhibition of adenylyl cyclase is the best characterised of the signal transduction pathways associated with cannabinoid receptor stimulation and anandamide is known to inhibit isoproterenol-induced accumulation of cyclic AMP (Childers & Deadwyler, 1996).

The aim of this series of experiments is to characterise the effects of cannabinoids on heart rate in the presence and absence of β-adrenoceptor tone.

3.2 Experimental Protocol

3.21 Agonist studies

All tissues were set up as described in 2.2.3. Agonists were introduced in a cumulative manner to naïve right atria in a tissue bath in order to construct concentration-response curves. (-)-Isoproterenol hydrochloride (isoprenaline) at a concentration of 0.01μM, where used, was added to the right atria prior to the addition of the agonist to cause an increase in heart rate. Bolus doses of agonist were added once the isoprenaline-induced increase in contractile rate had stabilised (5-10 minutes after addition). Enough time was allowed for each response to plateau before a subsequent agonist concentration was added. Only one cumulative concentration-response curve was constructed per individual preparation.

3.22 Antagonist studies

Antagonists or inhibitors (final concentration 1μM), where used, were added 30 minutes before agonist addition (cannabinoid or isoprenaline) to allow equilibration. Only one concentration-response curve was constructed per individual preparation.

3. 3 Drug preparation

The following drugs were used: Isoprenaline, capsazepine and indomethacin (all from Sigma, Gillingham, UK), anandamide and methanandamide (obtained as solutions of 5mg/ml dissolved in Tocrisolve^{TM} 100, a vehicle of 1:4 soya oil:water mixture emulsified with poloxamer F188), AM281, AM404, JWH-133 (Tocris Cookson Ltd, Bristol, UK) and SR141716A and SR144528 (gifts from Dr Barth of Sanofi-Aventis, Montpellier, France). Serial dilutions were made in Krebs bicarbonate solution in the

case of isoprenaline, anandamide and methanandamide. Ascorbic acid (approximately 1mM) was present in the stock solution of isoprenaline to prevent oxidation. AM281, AM404, SR141716A and SR144528 were first dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific, Leicestershire, UK) to give a stock concentration of 2mM followed by a 1:10 dilution in 50:50 DMSO to Krebs bicarbonate solution to give a final concentration of 1μM in the organ bath. Indomethacin was dissolved in ethanol (Fisher Scientific, Leicestershire, UK) to give a final concentration of 1μM in the organ bath.

3. 4 Statistical analysis

Responses are expressed as percentage of the resting rate or as a percentage of the increase induced by isoprenaline and are reported as mean ± S.E.M. Statistically significant ($P<0.05$) differences between results were assessed with ANOVA supported by Dunnett's multiple comparison test against a single control.

3.5 Results

3.51 Studies in naïve isolated right atria

3.51a Agonists Studies

Baseline rates did not significantly vary among any of the experimental groups (Table 3.1).

Tocrisolve™ 100 vehicle control administered at the equivalent amounts used to deliver cannabinoids had no significant effect on chronotropy in naïve right atria (0.6 ± 0.7% of baseline rate at the highest concentration). Addition of anandamide to naive right atria resulted in a small but significant increase in chronotropy. The increases seen were concentration-dependent with the maximum mean increase in chronotropy (11 ± 2% of baseline rate) induced by a concentration of $3x10^{-5}$M. No significant alteration in baseline chronotropy was observed with methanandamide at any concentration when compared to the vehicle control (Figure 3.1)

3.51b Antagonist Studies

None of the antagonists significantly affected baseline rate (Table 3.1).

Tocrisolve™ 100 vehicle control had no significant effect on chronotropy in naïve right atria in the presence of either DMSO (vehicle for SR141716A, SR144528, AM281, AM404 and capsazepine) or ethanol (vehicle for indomethacin, Figures 3.2 and 3.4 respectively).

Anandamide in the presence of either DMSO or ethanol significantly increased chronotropy by 7 ± 2% and 13 ± 2% of baseline rate respectively (Figures 3.2 and 3.4 respectively).

The presence of SR141716A (1μM), SR144528 (1μM), AM281 (1μM) or capsazepine (1μM), had no significant effect on the anandamide-induced positive chronotropy (Figure 3.3). However the presence of AM404 significantly inhibited anandamide-induced positive chronotropy (Figure 3.3). Anandamide-induced positive chronotropic responses were also significantly inhibited by the presence of 1μM indomethacin (Figure 3.4).

3.52 Studies using isoprenaline-treated isolated right atria

3.52a Agonist Studies

Addition of 0.01μM isoprenaline caused a significant increase in heart rate (150 ± 18% BPM of baseline levels, Table 3.2). Tocrisolve^{TM} 100 vehicle control had no significant effect on the chronotropy induced by isoprenaline (Figure 3.5).

In isoprenaline-treated right atria, anandamide caused a significant negative chronotropy (Figure 3.5). The highest concentration used ($3x10^{-5}$M) caused an 80% decrease of the isoprenaline-stimulated chronotropy. Methanandamide at the same concentration ($3x10^{-5}$M) also significantly decreased rate of atrial contraction by 46% of the isoprenaline-induced rate (Figure 3.5).

Addition of ACPA (selective CB_1 agonist) had no significant effect on chronotropy (Figure 3.5). However, the addition of JWH-133 (selective CB_2 agonist) significantly reduced chronotropy by 51% of the isoprenaline-induced rate (Figure 3.5) at the highest concentration used ($3x10^{-5}$M).

3.52b Antagonist Studies

None of the antagonists had a significant effect on baseline chronotropy (Table 3.3). Tocrisolve^{TM} 100 vehicle control had no significant effect on chronotropy in right atria pre-treated with isoprenaline in the presence of DMSO (vehicle for SR141716A, SR144528 and AM281; Figure 3.6).

In isoprenaline-treated right atria, in the presence of DMSO anandamide caused a significant negative chronotropy (Figure 3.6). The highest concentration used ($3x10^{-5}$M) caused a 38% decrease of the isoprenaline-stimulated heart rate.

The negative chronotropic response to anandamide in isoprenaline-treated right atria was not significantly affected by the presence of either SR141716A (1μM), AM281 (1 μM) or AM404 (1μM). However the presence of 1μM SR144528 significantly potentiated the negative chronotropic effect of anandamide, with the isoprenaline-induced rate being reduced by 90% of the isoprenaline-stimulated rate of atrial contraction. This was also the case for capsazepine where isoprenaline-induced rate was reduced to that of pre-isoprenaline-stimulated rates of atrial contraction (Figure 3.7).

Table 3.1 *Baseline contractile rate for naïve right atria treated with Tocrisolve™ 100, cannabinoid agonists, antagonists and the antagonist vehicle. Data expressed as mean ± S.E.M. $ signifies significant difference vs. baseline determined by Students unpaired t-test.*

	n	Baseline rate (BPM)	Baseline following addition of antagonist (BPM)	Mean maximal response (BPM)
Tocrisolve™ 100	6	287 ± 7		288 ± 8
Anandamide	11	290 ± 6		321 ± 7 $
Methanandamide	4	301 ± 5		308 ± 9
DMSO + Vehicle	4	310 ± 12	307 ± 7	303 ± 8
DMSO + Anandamide	5	311 ± 19	314 ± 16	334 ± 11
SR141716A + Anandamide	4	289 ± 7	294 ± 4	327 ± 14
AM281 + Anandamide	3	291 ± 11	300 ± 5	312 ± 13
SR144528 + Anandamide	6	272 ± 10	273 ± 8	302 ± 19
AM404 + Anandamide	7	308 ± 10	308 ± 6	319 ± 8
Capsazepine + Anandamide	3	279 ± 16	287 ± 13	307 ± 23
Ethanol + Tocrisolve™ 100	4	310 ± 8	322 ± 13	317 ± 14
Ethanol + Anandamide	4	292 ± 27	297 ± 19	335 ± 21
Indomethacin + Anandamide	7	313 ± 6	307 ± 5	323 ± 12

BPM = beats per minute.

Table 3.2 *Baseline contractile rate for right atria treated with 0.01 μM isoprenaline followed by treatment with Tocrisolve™ 100 or various cannabinoid receptor agonists. Data expressed as mean ± S.E.M. * signifies significant difference vs. baseline and $ signifies significant difference vs. isoprenaline-induced rate determined by Students unpaired t-test.*

	n	Baseline rate (BPM)	Isoprenaline-induced rate (BPM)	Mean maximal response (BPM)
Isoprenaline + Tocrisolve™ 100	4	299 ± 12	446 ± 9 *	446 ± 9
Isoprenaline + Anandamide	7	285 ± 12	426 ± 19 *	313 ± 15 $
Isoprenaline + Methanandamide	5	285 ± 7	453 ± 9 *	375 ± 25 $
Isoprenaline + ACPA	3	296 ± 17	461 ± 9 *	425 ± 10 $
Isoprenaline + JWH-133	4	290 ± 10	457 ± 19 *	268 ± 24 $

BPM = beats per minute.

Table 3.3 *Baseline contractile rate for right atria treated with 0.01 μM isoprenaline followed by treatment with agonist vehicle (Tocrisolve™ 100), antagonist vehicle (DMSO or Tocrisolve™ 100) or various combinations of cannabinoid receptor agonists and antagonists. Data expressed as mean ± S.E.M. * signifies significant difference vs. baseline and $ signifies significant difference vs. isoprenaline-induced rate determined by Students unpaired t-test.*

	n	Baseline rate (BPM)	Baseline following addition of antagonist (BPM)	Isoprenaline (BPM)	Mean maximal response (BPM)
DMSO + Isoprenaline + Tocrisolve™ 100	8	305 ± 5	304 ± 7	454 ± 4 *	451 ± 7
DMSO + Isoprenaline + Anandamide	4	289 ± 15	292 ± 10	434 ± 17 *	383 ± 34
SR141716A + Isoprenaline + Anandamide	5	288 ± 12	286 ± 12	468 ± 5 *	343 ± 35 $
AM281 + Isoprenaline + Anandamide	5	299 ± 8	306 ± 13	449 ± 13 *	416 ± 14
SR144528 + Isoprenaline + Anandamide	5	296 ± 12	313 ± 19	460 ± 9 *	336 ± 9 $
AM404 + Isoprenaline + Anandamide	5	284 ± 5	302 ± 9	448 ± 13 *	384 ± 25
Capsazepine + Isoprenaline + Anandamide	3	298 ± 10	292 ± 17	463 ± 8 *	282 ± 17 $

BPM = beats per minute.

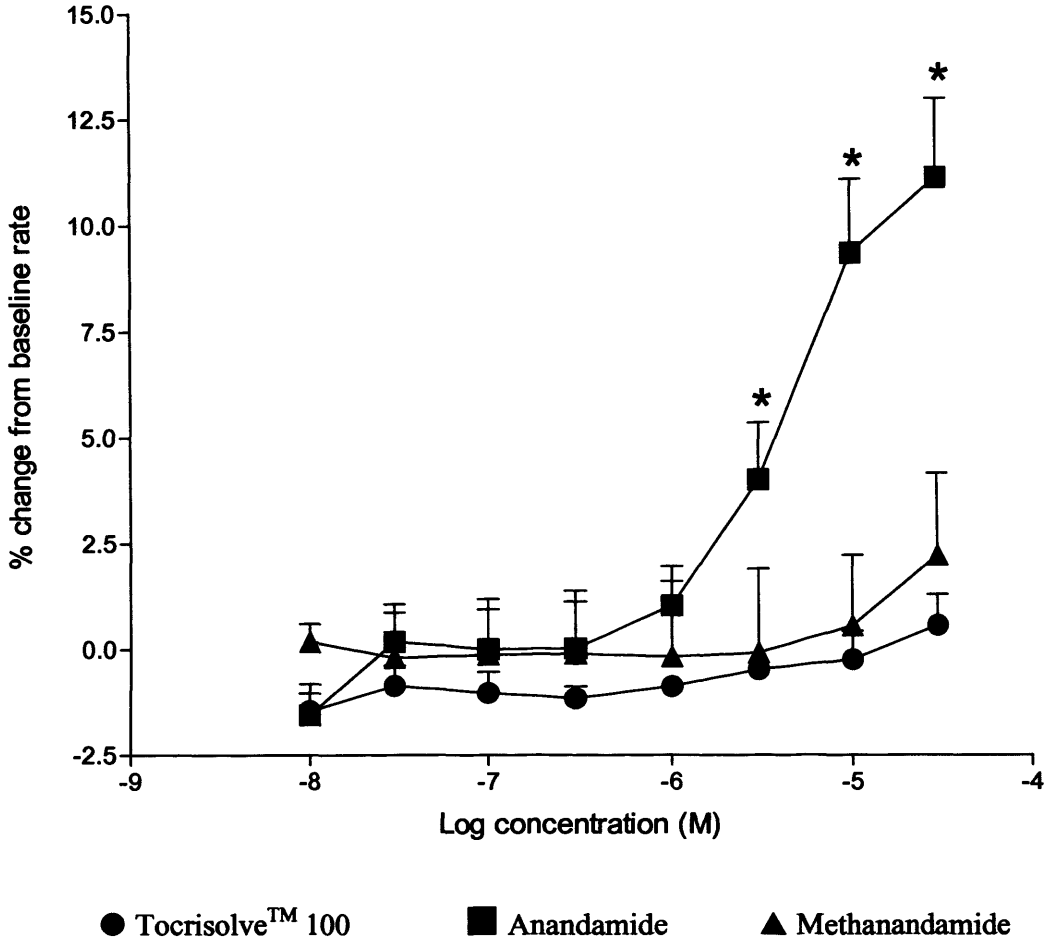


Figure 3.1 *Concentration response curves for the effect of anandamide (n = 11) and methanandamide (n = 4) on right atrial spontaneous rate. Effects of Tocrisolve™ 100 (vehicle for anandamide, n = 6) are shown for comparison. Error bars represent SEM. * P≤0.05 vs. vehicle control as determined by ANOVA supported by Dunnett's post-hoc test.*

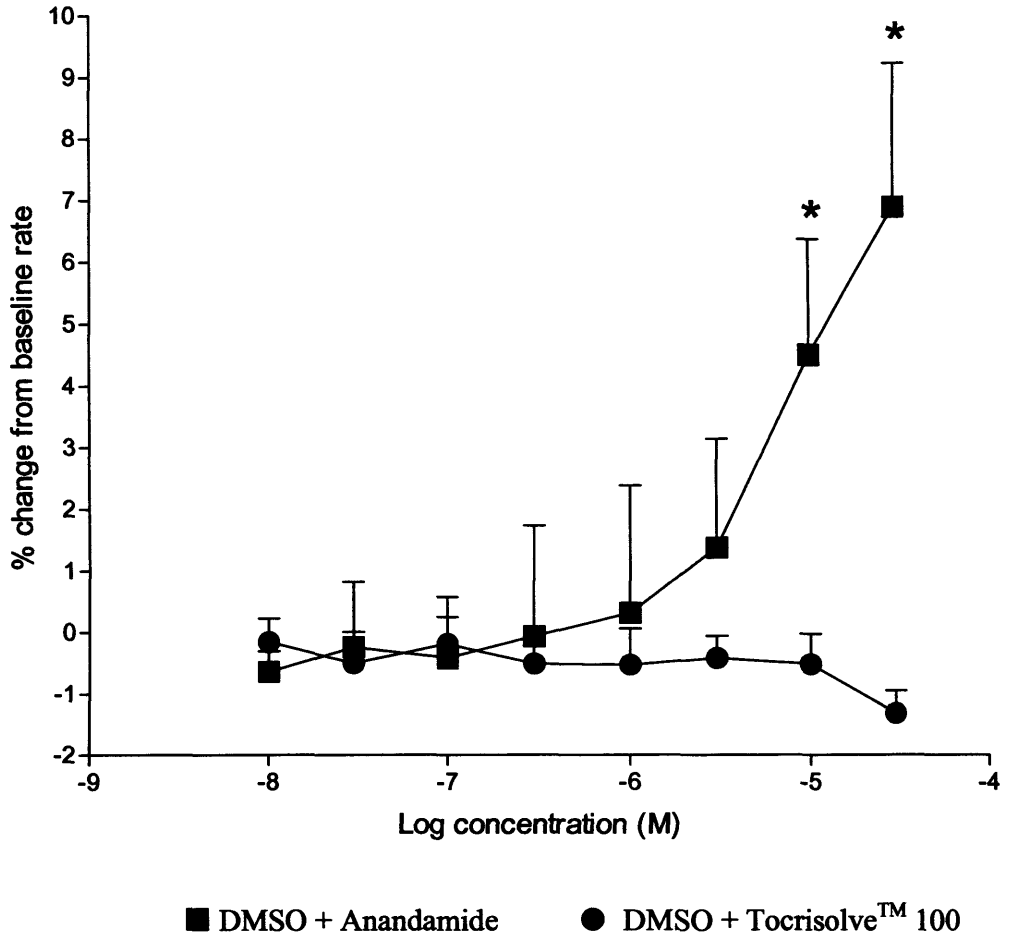


Figure 3.2 *Concentration response curve for the effect of anandamide (n = 5) on spontaneous rate in the presence of DMSO. The effects of Tocrisolve™ 100 (vehicle for anandamide) in the presence of DMSO (n = 4) added at equivalent concentrations to those used for anandamide is also shown for comparison. Error bars represent SEM. * $P \leq 0.05$ vs. vehicle control as determined by ANOVA supported by Dunnett's post-hoc test.*

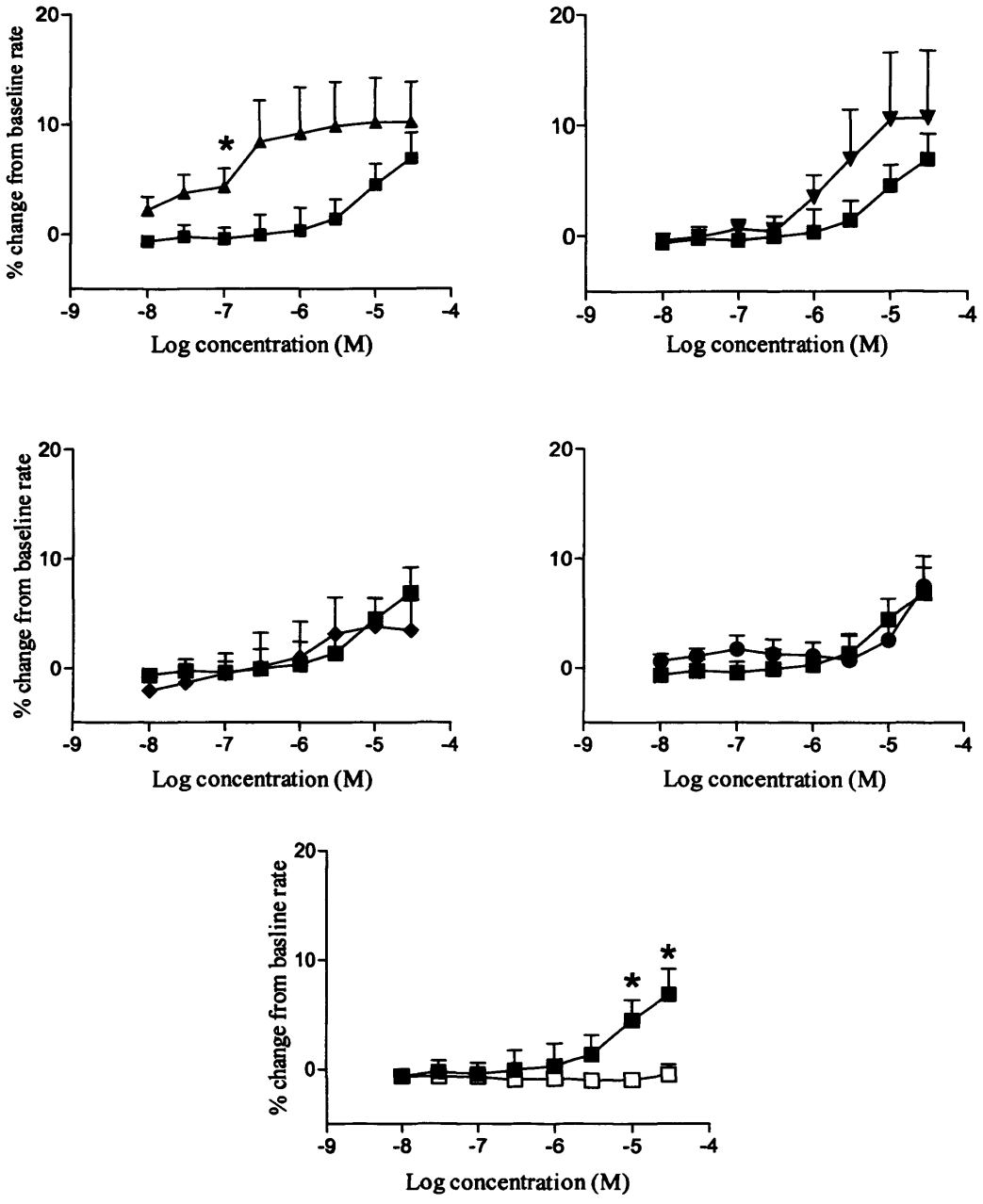


Figure 3.3 *Concentration response curves for the effect of anandamide on right atrial spontaneous rate in the absence (n = 5) and presence of 1μM SR141716A (n = 5), 1μM SR144528 (n = 6), 1μM AM281 (n = 3), 1μM capsazepine (n = 3) and 1μM AM404 (n = 7). The same anandamide group has been included on all graphs for ease of comparison. Error bars represent SEM. * P≤0.05 vs. anandamide as determined by ANOVA supported by Dunnett's post-hoc test.*

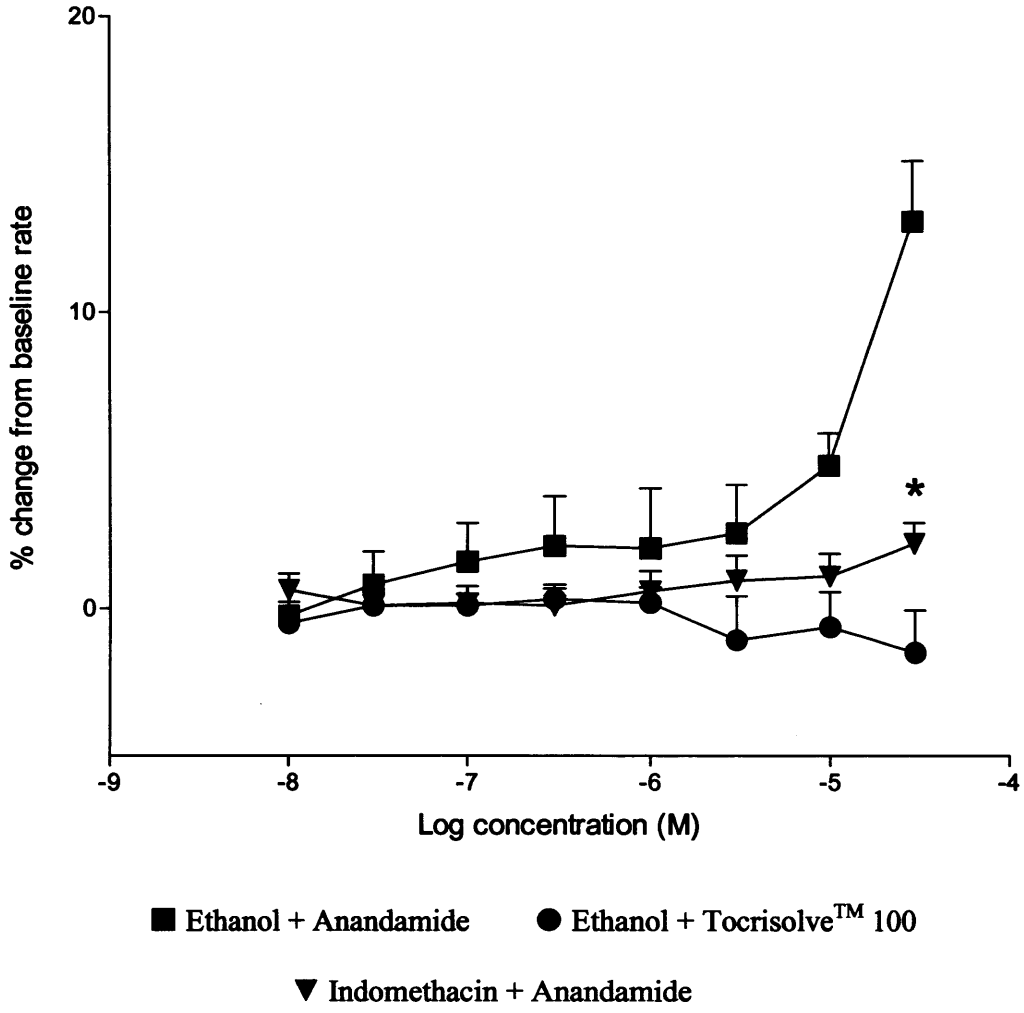


Figure 3.4 *Concentration response curves for the effect of anandamide on right atrial spontaneous rate in the presence of 1μM indomethacin (n = 4) and its vehicle (ethanol, n = 4). The effect of Tocrisolve™ 100 (vehicle for anandamide) in the presence of ethanol (n = 4) is also shown for comparison. Error bars represent SEM. * = P≤0.05 vs. ethanol + anandamide determined by ANOVA supported by Dunnett's post hoc test.*

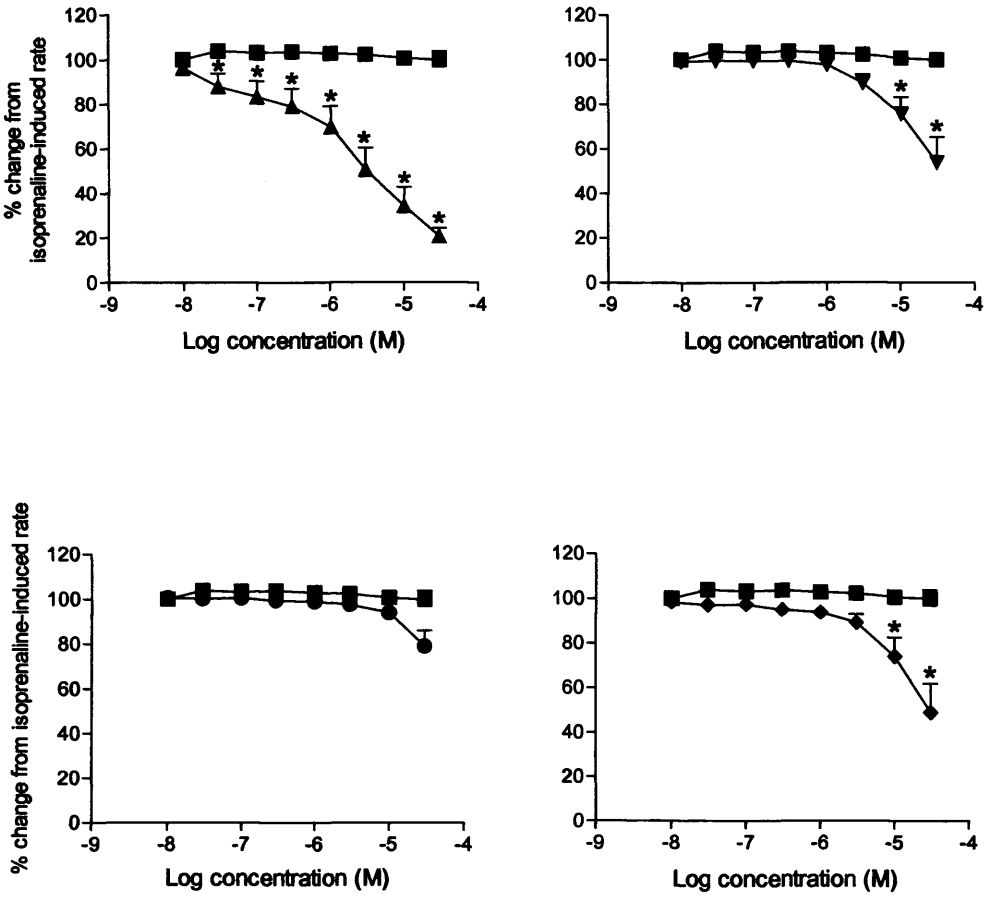


Figure 3.5 *Concentration response curve for the effect of the cannabinoid agonists anandamide (n = 7), methanandamide (n = 5), ACPA (n = 3) and JWH-133 (n = 4) on right atrial contractile rate stimulated by 0.01 μM isoprenaline. The effect of Tocrisolve™ 100 (n = 4) is also shown for comparison. Error bars represent SEM. * = P≤0.05 vs. Isoprenaline + Tocrisolve™ 100 as determined by ANOVA supported by Dunnett's post hoc test.*

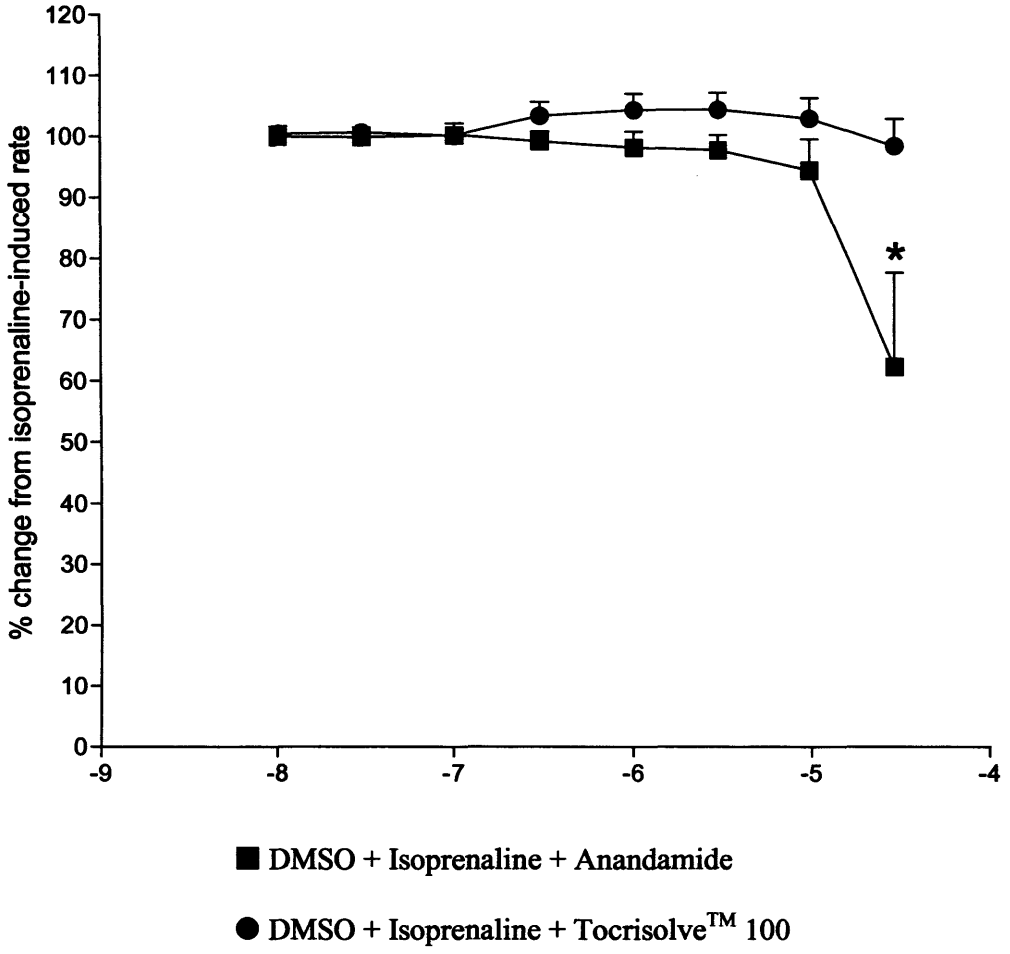


Figure 3.6 *Concentration response curve for the effect of anandamide (n = 4) on right atrial contractile rate stimulated by 0.01 μM isoprenaline. Effects of Tocrisolve™ 100 (n = 8) are shown with DMSO and isoprenaline for comparison. Error bars represent SEM. * = P≤0.05 vs. DMSO + Isoprenaline + Tocrisolve™ 100 as determined by ANOVA supported by Dunnett's post hoc test.*

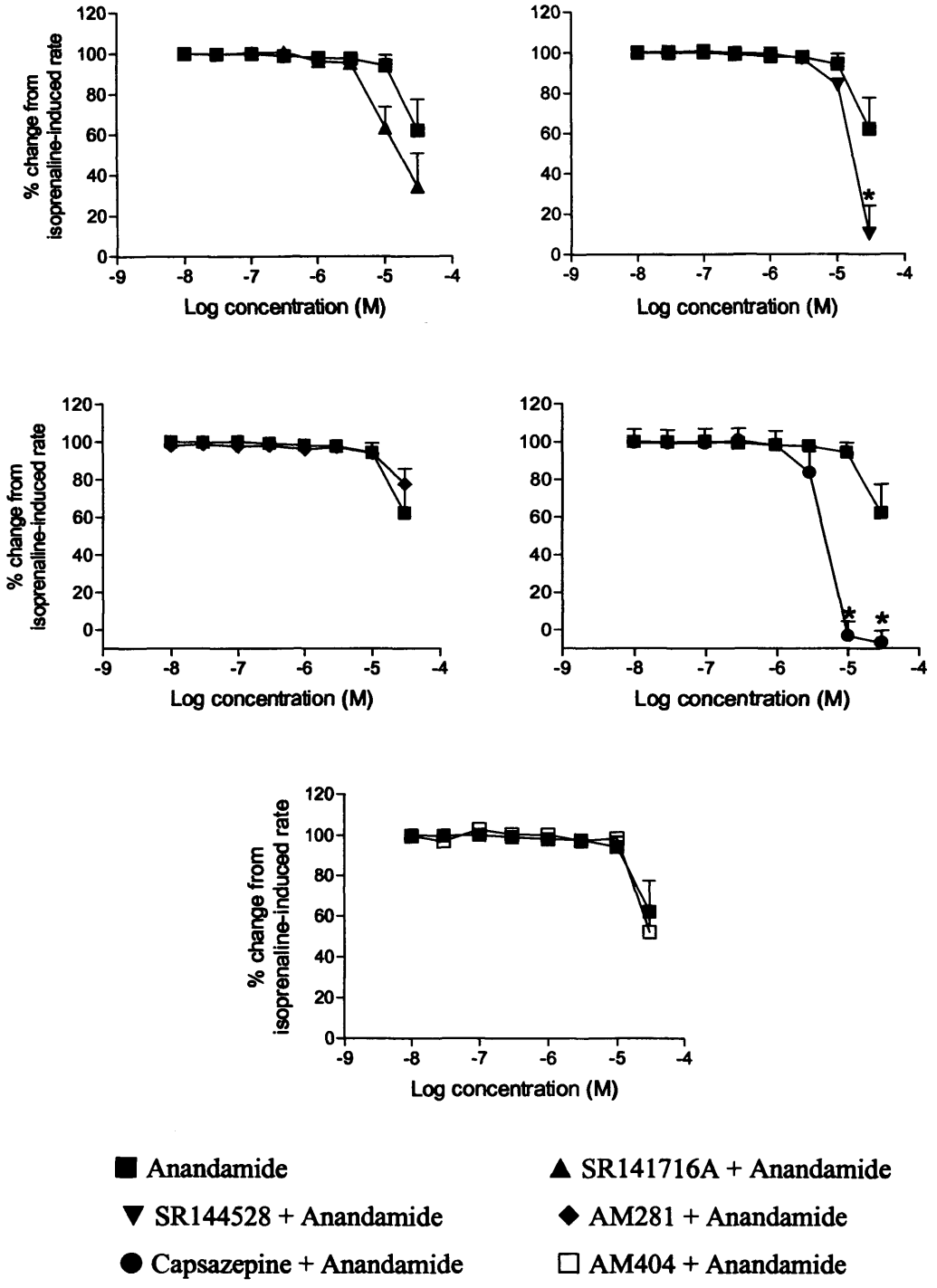


Figure 3.7 *Concentration response curve for the effect of anandamide on right atrial contractile rate stimulated with 0.01 μM isoprenaline in the absence (n = 4) and presence of 1μM SR141716A (n = 5), 1μM SR144528 (n = 5), 1μM AM281 (n = 5), 1μM capsazepine (n = 3) and 1μM AM404 (n = 5). Error bars represent SEM. * = P≤0.05 vs. anandamide as determined by ANOVA supported by Dunnett's post hoc test.*

3.6 Discussion

The main findings of this study were that anandamide elicited either positive or negative chronotropic responses depending upon the state of the atria. In naïve atria, anandamide causes a small, but significant positive chronotropy whilst in atria pre-treated with isoprenaline, negative chronotropic responses were observed. These findings demonstrate that responses to cannabinoids depend upon the presence or absence of β-adrenoceptors tone and may go someway to explaining the lack of concordance when the effects of cannabinoids on heart rate are studied *in vivo*.

The data presented shows that anandamide caused a significant increase in chronotropy of isolated spontaneously beating right atria. This result demonstrates that in addition to previously reported activities on the central and peripheral nervous system (see reviews Felder & Glass, 1998; Howlett *et al.*, 2002) anandamide can alter heart rate at the level of the sino-atrial node rather than altering central or peripheral nervous output. However, the biological significance of this response is questionable as the increase in heart rate is relatively small ($11 \pm 2\%$ of baseline rate).

The positive chronotropic response to anandamide was not blocked by either of the classic cannabinoid receptor antagonists SR141716A (CB_1 receptor antagonist), or SR144528 (CB_2 receptor antagonist). However, AM404 the anandamide transporter inhibitor (Beltramo *et al.*, 1997; Khanolkar & Makriyannis, 1999), reduced the positive chronotropy caused by anandamide providing evidence that the site of action for anandamide is intracellular. It was also found that anandamide-induced positive chronotropy was inhibited by indomethacin (a cyclooxygenase inhibitor) indicating that

anandamides mechanism of action involved the formation of products of the cyclooxygenase pathway such as prostaglandins or prostacyclins (Belton & Fitzgerald, 2003). Metabolism of anandamide by COX-2 (an inducible form of cyclooxygenase) has been shown to produce a novel class of prostaglandin (Yu *et al.*, 1997), however, the biological significance of these pathways has yet to be discovered. There are a number of theories presently held about their significance. One suggests that oxygenation of anandamide may be an inactivation pathway. Another is that it is an activation pathway in which the metabolic stability of the products are increased above that of the parent endocannabinoids as the products of cyclooxygenase metabolism are less labile than the parent, therefore cyclooxygenase metabolism acts to increase the duration of the response. A third theory is that the oxygenated products of COX-2 metabolism of cannabinoids are unique signal transduction mediators. Finally that oxidised endocannabinoids may serve as pro-drugs as the prostanoids produced are more metabolically stable than free-acid prostaglandins therefore having a longer half life to act as systemic mediators or pro-drugs (Kozak *et al.*, 2004; Patrignani *et al.*, 2005).

Methanandamide, which in previous studies was shown to have similar effects to anandamide in the heart, *i.e.* the triphasic effect (Malinowska *et al.*, 2001), did not elicit the positive chronotropic effect seen with anandamide. This supports the finding that anandamide mediates positive chronotropy by uptake and release of products of cyclooxygenase as methanandamide is a non-hydrolysable analogue of anandamide (Abadji *et al.*, 1994) and therefore insensitive to metabolism by cyclooxygenase.

Paradoxically, anandamide reduced the rate of atrial contraction once chronotropy had been increased with isoprenaline. This negative chronotropic response, revealed by isoprenaline treatment, was mimicked by methanandamide and therefore independent of cyclooxygenase metabolism unlike the previously observed positive chronotropic response. Thus, cannabinoids appear to mediate opposing actions on contractile rate mediated by the sino-atrial node depending upon β-adrenoceptor tone.

As the negative chronotropy induced by anandamide in the presence of isoprenaline was not due to release of cyclooxygenase products, the possibility that the response was mediated by activation of one of the classical cannabinoid receptors was investigated using agonists and antagonists selective for either CB_1 or CB_2 receptors. ACPA the selective CB_1 agonist (Hillard *et al.*, 1999) had no effect on isoprenaline-stimulated atrial contraction rate. However, treatment with JWH-133 a CB_2 selective agonist (Huffman *et al.*, 1999) resulted in a negative chronotropic response similar in magnitude to that caused by anandamide or methanandamide. This suggests that the negative chronotropy seen with anandamide and methanandamide in the presence of isoprenaline is mediated via CB_2 receptors.

If the anandamide-mediated negative chronotropy was the result of CB_2 receptor activation it would be expected that SR144528 (selective CB_2 antagonist) would have blocked this response. However, this was not the case as the presence of SR144528 significantly potentiated the negative chronotropic effect. This finding would suggest that CB_2 receptor activation causes an increase in heart rate and by blocking this response using SR144528 reveals a functional antagonism resulting in the potentiation

of the negative chronotropy. This data clearly does not fit with that obtained using JWH-133 where negative chronotropy was induced. A possible explanation for this could be that SR144528 was not used at a sufficiently high concentration to block CB_2 receptors. However, we used a concentration of 1μM which has been previously shown to effectively antagonise responses mediated by CB_2 receptors (Rinaldi-Carmona *et al.*, 1998).

AM404 did not significantly affect the negative chronotropic effect of anandamide. This result suggests that the site of action for cannabinoid-mediated negative chronotropy in the presence of isoprenaline is not intracellular, which confirms the results seen in the presence of methanandamide.

Another receptor which could be involved in the negative chronotropic effect of anandamide is TRPV1. However, the TRPV1 antagonist capsazepine did not block the negative chronotropy seen, in fact; the presence of this antagonist potentiated the effect. This is similar to that seen in the presence of SR144528 and again could be due to activation of TRPV1 causing an increase in heart rate and thus by blocking this response using capsazepine we revealed a functional antagonism resulting in the potentiation of the negative chronotropy.

It would appear that at least two mechanisms are involved in the regulation of heart rate. One, which increases (positive chronotropy) and another, which decreases (negative chronotropy) heart rate. Physiologically, the latter effect would be observed upon sympathetic stimulation. Isoprenaline activates adenylate cyclase leading to increased

intracellular levels of the secondary messenger, cyclic AMP, that mediates an increase in heart rate (Whalen & Lewis, 1999). Anandamide is known to inhibit isoproterenol-induced accumulation of cyclic AMP (Childers & Deadwyler, 1996). This has prompted the notion that there is a novel G-protein-coupled receptor for cannabinoids distinct from the neuronal CB_1 receptor because inhibition of the isoproterenol-induced accumulation of cyclic AMP was insensitive to CB_1 antagonists (Sagan *et al.*, 1999). Investigation into this novel receptor has focused on neurons and astrocytes, where the same agonist profile has been demonstrated but it was only in the astrocytes where SR141716A did not block the inhibition of the isoproterenol-induced accumulation of cyclic AMP by anandamide. This novel receptor has been shown to be more abundant in astrocytes than in neurons as specific binding sites for [^{3}H]-SR141716A and staining with a polyclonal CB_1 receptor antibody was only observed in neurons not astrocytes (Sagan *et al.*, 1999). As cannabinoids opposed the increase in rate of contraction induced by isoprenaline at an intracellular site, it is possible that cannabinoids might inhibit the activity of adenylyl cyclase or some other downstream component of the β-adrenoceptor transduction pathway. It could be speculated that addition of anandamide to heart tissues would inhibit cyclic AMP and the rate of contraction would diminish as was shown by a decrease in heart rate.

Both CB_1 and CB_2 receptor protein has been found in rat hearts using western blot analysis with rabbit polyclonal antibodies (Bouchard *et al.*, 2003), however, prior to this it had been reported that there was a lack of CB_2 mRNA in the rat heart (Brown *et al.*, 2002). The data presented shows that functionally, neither CB_1 nor CB_2 are involved in the positive chronotropic effect of anandamide on naïve right atria because neither CB_1

receptor antagonists (SR141716A and AM281) nor a CB_2 receptor antagonist (SR144528) significantly inhibited anandamide-induced positive chronotropy. However, the selective CB_2 agonist, JWH-133 (Pertwee, 1999) caused the negative chronotropic effect seen with anandamide in right atria pre-treated with isoprenaline again suggesting two different pathways in the involvement of cannabinoids on the heart.

This investigation demonstrates that cannabinoids elicit a number of responses in the cardiovascular system which are independent of the influences of the central and peripheral nervous system and that these are due to separate mechanisms of action. This study also shows that the response to cannabinoids depends upon the state of the tissue. For example in this study anandamide has been shown to fine tune heart rate by increasing the rate all be it by a small amount in the absence of sympathetic tone and also reducing the rate in its presence.

Chapter 4

Investigation into the cardioprotective role of endocannabinoids in a model of global ischaemia

Table of Contents

4.1 Introduction

Two studies indirectly implicate endocannabinoids in the protection against myocardial ischaemia-reperfusion injury. One reports the involvement of cannabinoids in protection afforded by lipopolysaccharide treatment (Lagneux & Lamontagne, 2001) and the other implicates endocannabinoids in the limitation of infarction induced by heat stress preconditioning (Joyeux *et al.*, 2002). In both studies SR144528 the CB_2 receptor antagonist inhibited the protective effects of the treatments indicating that activation of the CB_2 receptor is involved.

CB_2 receptor involvement in cardioprotection has further been reported upon in a study by Lepicier *et al.* (2003). In that study palmitoylethanolamide and 2-arachidonoylglycerol but not anandamide were shown to improve recovery and reduce infarct size in a low-flow model of global ischaemia. A selective CB_1 agonist, ACEA and a selective CB_2 agonist, JWH-015 also limited infarction. The results from this study are confusing because palmitoylethanolamide is a weak ligand at both the cannabinoid receptors (Lambert *et al.*, 1999) and was shown to be devoid of any activity when administered to isolated rat hearts (Ford *et al.*, 2002).

The pharmacology of 2-arachidonoylglycerol and anandamide are very similar as both bind and stimulate the CB_1 and CB_2 receptors (Steffens *et al.*, 2005; Sugiura *et al.*, 2000). Therefore the difference seen between these endocannabinoids in the Lepicier study are unlikely to be due to contrasting affinities or efficacies at the cannabinoid receptors particularly as protection could be induced by the selective agonists. It has been suggested that anandamide may not have been effective because it was rapidly

degraded, however, this is unlikely to be the case as both anandamide and 2-arachidonoylglycerol are sensitive to uptake and metabolism (Goparaju *et al.*, 1998; Laine *et al.*, 2002) therefore differences are unlikely to be due to different rates of degradation.

Given the confusion about the effectiveness of endocannabinoids in mediating cardioprotection we decided to assess whether anandamide could limit infarct size induced by ischaemia-reperfusion injury measured directly using tetrazolium chloride in rat isolated hearts and identify the pharmacological mechanism.

4.2 Experimental protocols

A constant pressure Langendorff buffered-perfused heart preparation was used with rat hearts undergoing a period of global ischaemia. A detailed description of this preparation can be found in section 2.3. In brief, following attachment of the aorta to the cannula and immersion of the heart into the organ bath, hearts were aerobically perfused for the first 15 minutes (baseline values taken at 10 minutes and pre-ischaemic values at 15 minutes). 5 minutes prior to the ischaemia hearts were infused with either vehicle or agonist at 10% of the flow rate. Hearts then underwent a 30 minute period of no-flow global ischaemia induced by clamping the perfusion line to the heart.

Vehicle or agonists were infused (at 10% of the flow rate) at the same time as reperfusion commenced and continued throughout the following 2 hours of reperfusion. Figure 4.1 shows the experimental protocol used for these experiments.

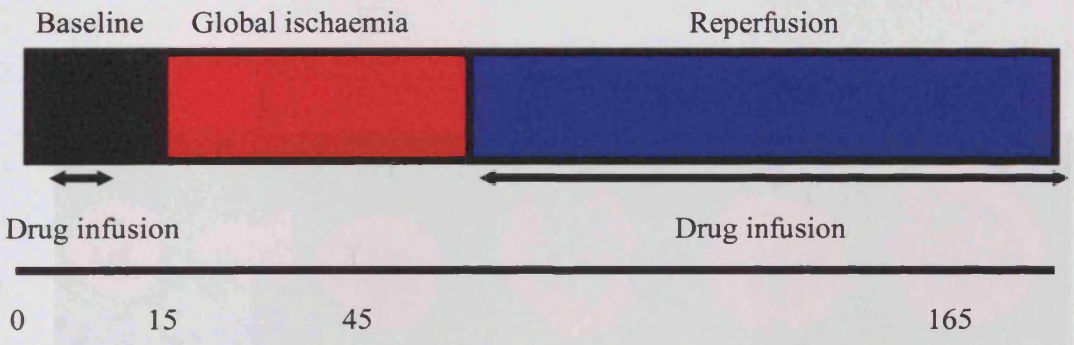


Figure 4.1 *Diagrammatic representation of the global ischaemia protocol. Hearts were aerobically perfused for the first 15 minutes (black). Baseline values were taken at 10 minutes and pre-ischaemic values were taken just before ischaemia was induced at 14 minutes. Reperfusion commenced after 30 minutes of global, no-flow ischaemia (red). Hearts were reperfused for 2 hours (blue) and then frozen. Reperfusion values were reported at the end of the protocol, before hearts were removed and frozen ready for tetrazolium chloride staining. The arrows indicate the infusion of agonists or their vehicle. Antagonists or their vehicle (DMSO) where used, were present throughout the protocol.*

At the end of reperfusion the intraventricular balloon was deflated and removed. The hearts were then cut from the cannula, frozen and sliced into 5 or 6 transverse sections approximately 4mm thick with a single edged razor. The slices were then placed in a beaker containing a 1% solution of triphenyltetrazolium chloride for 5-10 minutes until a brick red colour had developed. Stained hearts were then stored in a 10% formalin solution (formaldehyde solution, phosphate buffered, pH 7.3) for 24 hours. The right ventricle and connective tissues were then removed. The stained slices were flattened between two glass plates separated by 2.5mm. Areas with an absence of red stain (infarcted area) were traced onto an acetate sheet by an investigator blinded to the experimental protocol. Acetate tracings were scanned into a PC running SigmaScan Pro 5 (Hounslow, UK) and the areas (in pixels) of infarction or viable tissue calculated. Infarct size is reported as the area unstained by triphenyltetrazolium chloride expressed as a percentage of the area at risk. Figure 4.2 shows a typically stained heart.

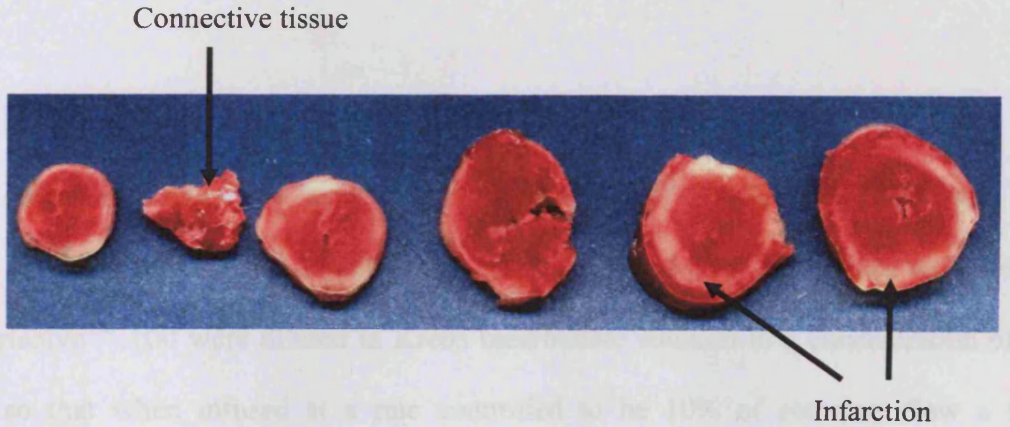


Figure 4.2 *Picture showing a typical example of the sections of a stained heart*

4.21 Agonist Studies

Hearts were randomly assigned to receive the cannabinoid vehicle, Tocrisolve™ 100 (a vehicle of 1:4 soya oil:water mixture emulsified with poloxamer F188, Tocris Cookson Ltd, Bristol, UK), or one of the following cannabinoid agonists (1μM): anandamide, methanandamide, HU-210, arachidonlycyclopropylamide (ACPA), JWH-133 or a combination of ACPA and JWH-133. Cannabinoid agonists or Tocrisolve™ 100 vehicle were infused at a flow rate controlled to be 10% of coronary flow.

4.22 Antagonist studies

Where used, antagonists were present in the perfusate throughout the protocol. Antagonists were prepared in dimethyl sulfoxide (DMSO) as a 1mM stock solution before being added to the perfusate (final concentration 1μM).

Hearts were randomised to one of three groups. The first consisted of the antagonist vehicle DMSO (0.01% vol.vol-1). The second group was treated with SR141716A and the third group with SR144528.

4.3 Drug preparations

The cannabinoid agonists, anandamide, methanandamide, ACPA, JWH-133 and HU-210 (Tocris Cookson Ltd, Bristol, UK) obtained as solutions of 5mg/ml dissolved in Tocrisolve™ 100 were diluted in Krebs bicarbonate solution to a concentration of 10^{-5}M so that when infused at a rate controlled to be 10% of coronary flow a final concentration of 1μM was obtained. The cannabinoid agonist vehicle Tocrisolve™ 100 was diluted in Krebs bicarbonate to give a concentration of 0.07% solution that when infused at 10% of coronary flow a final concentration of 0.007% was obtained.

SR141716A and SR144528 (gifts from Dr Barth of Sanofi-Aventis, Montpellier, France) were prepared in DMSO (Fisher Scientific, Leicestershire, UK) as 1mM stock solutions and added to the perfusate (final concentration was 1μM).

4.4 Statistical analysis

All data are expressed as the mean ± SEM of *n* experiments. Unpaired data were compared using an unpaired Student's t-test. Multiple comparisons against a single control were made using analysis of variance (ANOVA) followed by Dunnet's *post hoc* test (GraphPad InStat 3.0, California, USA). ANOVA with repeated measures was used to compare time course data (SPSS Inc., Chicago, USA). Statistical significance was taken at $P < 0.05$.

4.5 Results

4.51 Agonists Studies

Diastolic pressure, left ventricular developed pressure and coronary flow did not significantly vary among any of the experimental groups before ischaemia (Table 4.1).

In vehicle treated (Tocrisolve™ 100) control hearts, diastolic pressure, markedly elevated at the beginning of reperfusion, fell steadily throughout reperfusion and remained significantly elevated compared to pre-ischaemic values by the end of reperfusion (Figure 4.3). Left ventricular developed pressure recovered incompletely during reperfusion recovering to a maximum of 40 ± 6% of pre-ischaemia values after 50 minutes of reperfusion. After 50 minutes of reperfusion there was a steady decline in left ventricular developed pressure until the end of reperfusion such that by the end of reperfusion, the values were 29 ± 6% of pre-ischaemic values (Figure 4.3 and Table 4.1). Recovery of coronary flow during reperfusion was also incomplete reaching a maximum (37 ± 7% or pre-ischaemic values) after 20 minutes of reperfusion then declining to 24 ± 6% of pre-ischaemic values by the end of reperfusion (Figure 4.3 and Table 4.1). Infarct size, measured at the end of reperfusion was 26 ± 3% of the left ventricle (Figure 4.4 and Table 4.1).

None of the cannabinoid agonists used in the study had a significant effect on the recoveries of diastolic pressure, left ventricular developed pressure or coronary flow compared to control hearts (Figure 4.3 and Table 4.1). However, infarct size was significantly reduced by the presence of anandamide (10 ± 1% of the left ventricle) and methanandamide (12 ± 4% of the left ventricle) compared to hearts which received

vehicle (Figure 4.4 and Table 4.1). Infarct size in hearts treated with ACPA (26 ± 5% of the left ventricle) or JWH-133 (20 ± 2% of the left ventricle) individually or in combination (27 ± 4% of the left ventricle) were not significantly different from those obtained in vehicle control hearts (Figure 4.4 and Table 4.1).

4.52 Antagonist studies

Diastolic pressure, left ventricular developed pressure and coronary flow did not significantly vary among any of the experimental groups before ischaemia (Table 4.2).

In vehicle (Tocrisolve^{TM} 100) control hearts with 0.01% vol.vol^{-1} DMSO present, diastolic pressure was markedly elevated at the beginning, falling steadily throughout but remaining elevated by the end of reperfusion (Figure 4.5). Recovery of left ventricular developed pressure was incomplete reaching a maximum of 33 ± 8% of pre-ischaemic values after 40 minutes of reperfusion then falling to 18 ± 4% of pre-ischaemic values by the end of reperfusion (Figure 4.5). Coronary flow was impaired during reperfusion recovering to a maximum of 33 ± 7% of pre-ischaemic values after 20 minutes of reperfusion then falling to 17 ± 1% of pre-ischaemic values by the end of reperfusion (Table 4.2). Measured at the end of reperfusion, 28 ± 3% of the left ventricle was infarcted (Figure 4.8 and Table 4.2).

The recoveries of diastolic pressure, left ventricular developed pressure and coronary flow were not significantly different from vehicle (Tocrisolve^{TM} 100) control hearts treated with 0.01% vol.vol^{-1} DMSO in hearts where SR141716A (1μM, Figure 4.6) or SR144528 (1μM, Figure 4.7) were present. Infarcts obtained in hearts where

SR141716A or SR144528 were present (Figure 4.8 and Table 4.2) were similar to those in their absence.

Infusion of anandamide in the presence of 0.01% vol.vol^{-1} DMSO had no significant effect on the recovery of diastolic pressure, left ventricular developed pressure or coronary flow during reperfusion compared to vehicle control hearts (Figure 4.6 and Table 4.2). However infarct size, measured at the end of reperfusion, was significantly reduced to 12 ± 2% of the left ventricle (Figure 4.8 and Table 4.2).

The combination of SR141716A or SR144528 with anandamide infusion had no significant effect on recoveries of diastolic pressure, left ventricular developed pressure or coronary flow during reperfusion compared to SR141716A or SR144528 with the vehicle control (Tocrisolve^{TM} 100; Figures 4.6 and 4.7 and Table 4.2). However, the infarct size reduction observed when anandamide was infused alone was lost in the presence of either SR141716A or SR144528 (Figure 4.8 and Table 4.2).

Table 4.1 *Cardiac function and infarct size in hearts treated with cannabinoid agonists.*

	Perfusion Time (min)	*n*	Diastolic pressure (mmHg)	LVDP (mmHg)	CF ($ml.min^{-1}$)	Infarct size (% of LV)
Tocrisolve™ 100	10 15 135	13	9±1 8±1 41±9	106±8 100±6 19±3	15±1 14±1 2±1	26±3
Anandamide (1μM)	10 15 135	7	6±1 5±2 41±13	104±10 111±11 27±3	16±1 18±1 5±1	10±1
mAEA (1μM)	10 15 135	6	9±2 10±1 27±5	95±20 92±16 21±5	12±1 14±1 3±1	12±4
ACPA (1μM)	10 15 135	6	7±2 11±2 34±12	91±13 87±10 22±2	12±1 13±1 4±1	26±5
JWH-133 (1μM)	10 15 135	8	8±1 11±1 33±5	83±10 89±16 20±4	13±1 13±1 2±1	20±2
ACPA + JWH-133 (1μM each)	10 15 135	9	13±1 12±2 53±6	93±8 94±6 16±3	12±1 13±1 4±1	27±4

LVDP = left ventricular developed pressure, CF = coronary flow, LV = left ventricle and mAEA = methanandamide. Absolute values are given as mean ± s.e.m. with *n* the number of hearts in the group.

Table 4.2 *Cardiac function and infarct size in hearts treated with cannabinoid agonists and in the presence of the cannabinoid antagonists.*

	Perfusion Time (min)	*n*	Diastolic pressure (mmHg)	LVDP (mmHg)	CF ($ml.min^{-1}$)	Infarct size (% of LV)
Tocrisolve™ 100	10 15 135	7	10±1 9±2 41±11	101±10 96±9 18±5	13±2 13±1 2±1	28±3
Anandamide (1μM)	10 15 135	7	7±1 7±2 51±9	100±11 103±11 22±3	15±1 17±1 4±1	12±3
SR141716A (1μM)	10 15 135	6	16±2 16±2 56±6	88±6 82±5 15±3	12±2 12±2 2±1	18±2
SR141716A +anandamide (1μM)	10 15 135	6	12±1 12±1 53±6	81±3 81±5 22±1	13±1 13±1 3±1	20±4
SR144528 (1μM)	10 15 135	6	9±2 12±1 58±9	113±9 108±8 14±3	15±1 14±1 5±2	23±3
SR144528 + anandamide (1μM)	10 15 135	8	9±1 8±1 22±8	98±5 100±5 26±5	18±1 18±1 5±1	22±2

LVDP = left ventricular developed pressure, CF = coronary flow, LV = left ventricle and mAEA = methanandamide. Absolute values are given as mean ± s.e.m. with *n* the number of hearts in the group.

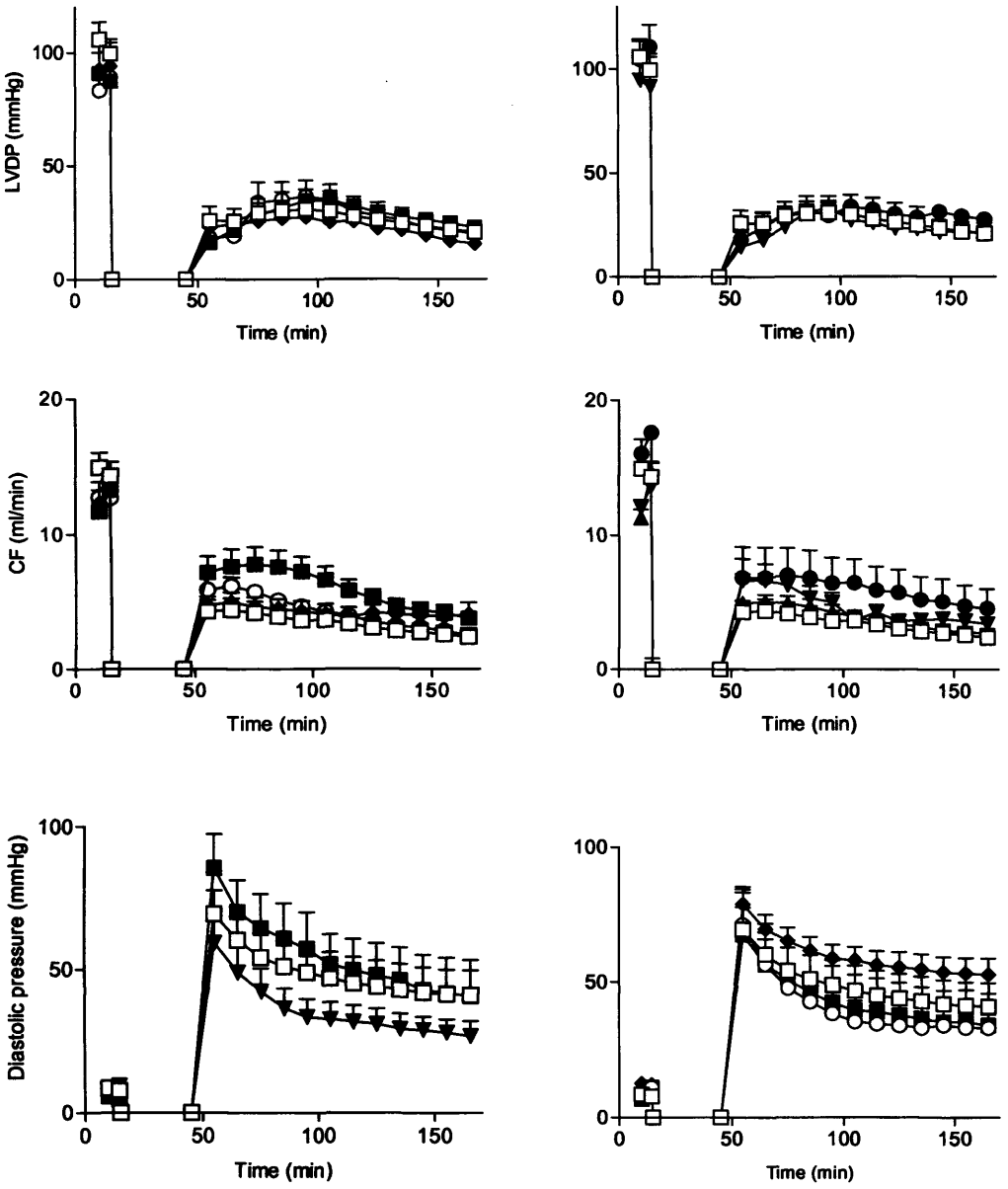


Figure 4.3 *Left ventricular developed pressure (LVDP), coronary flow (CF) and diastolic pressure for hearts treated with Tocrisolve™ 100 (control, □, n = 13) or 1μM anandamide (AEA, ●, n = 7), 1μM methanandamide (mAEA, ▼, n = 6), 1μM ACPA (■, n = 6), 1μM JWH-133 (○, n = 8), and a combination of 1μM ACPA and 1μM JWH-133 (◆, n = 8). The same control group appears in all panels. Left ventricular developed pressure, diastolic pressure and coronary flow did not significantly vary among any of the experimental groups (ANOVA with repeated measures of analysis).*

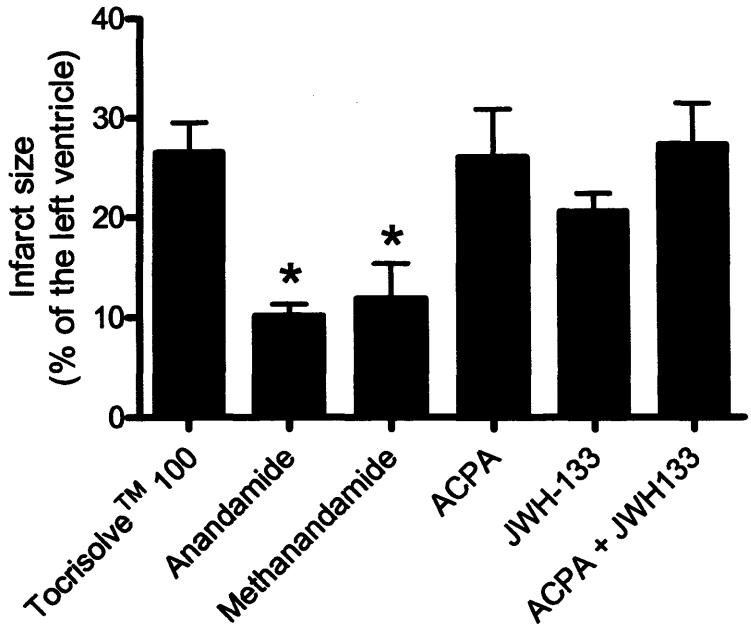


Figure 4.4 *Infarct sizes for individual hearts treated with Tocrisolve™ 100 (n = 13) or 1μM anandamide (n = 7), 1μM methanandamide (n = 6), 1μM ACPA (n = 6), 1μM JWH-133 (n = 8), and a combination of 1μM ACPA and 1μM JWH-133 (n = 9). * = P<0.05 vs. control (ANOVA supported by Dunnett's post hoc test).*

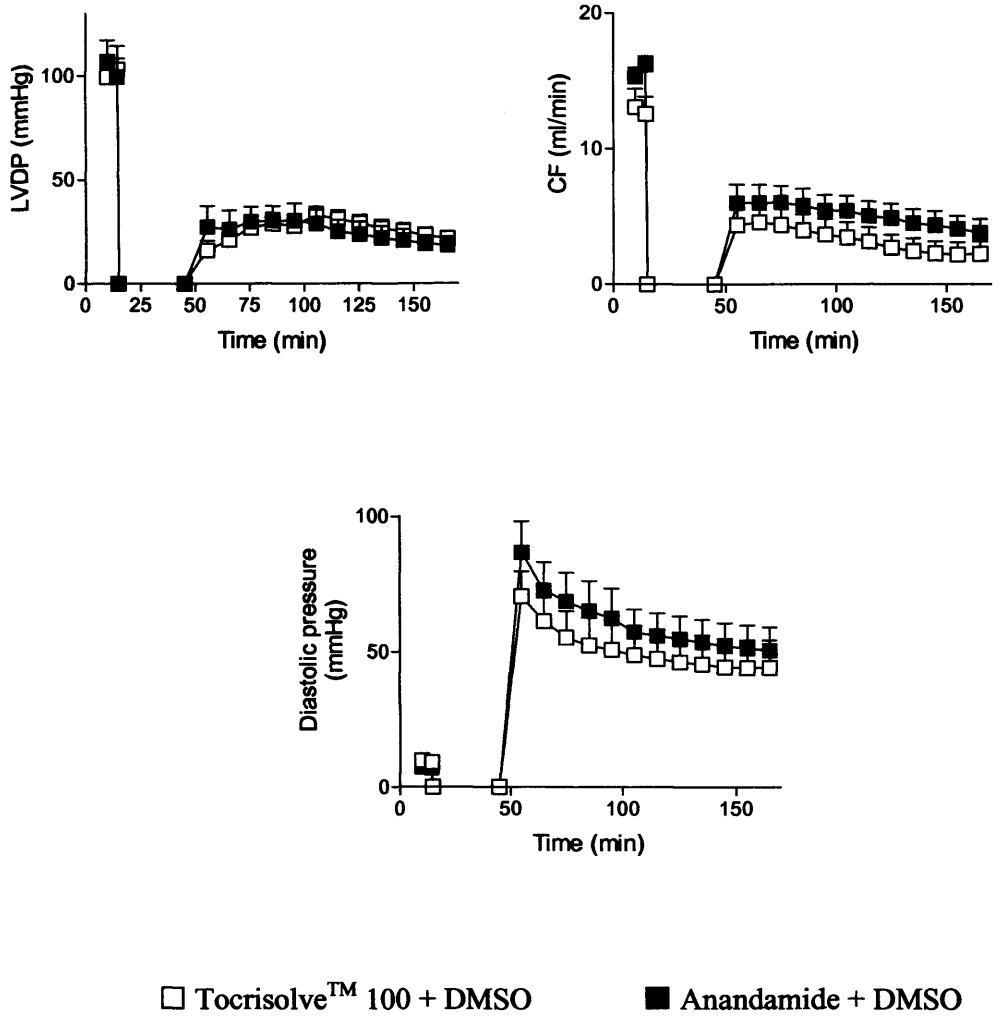
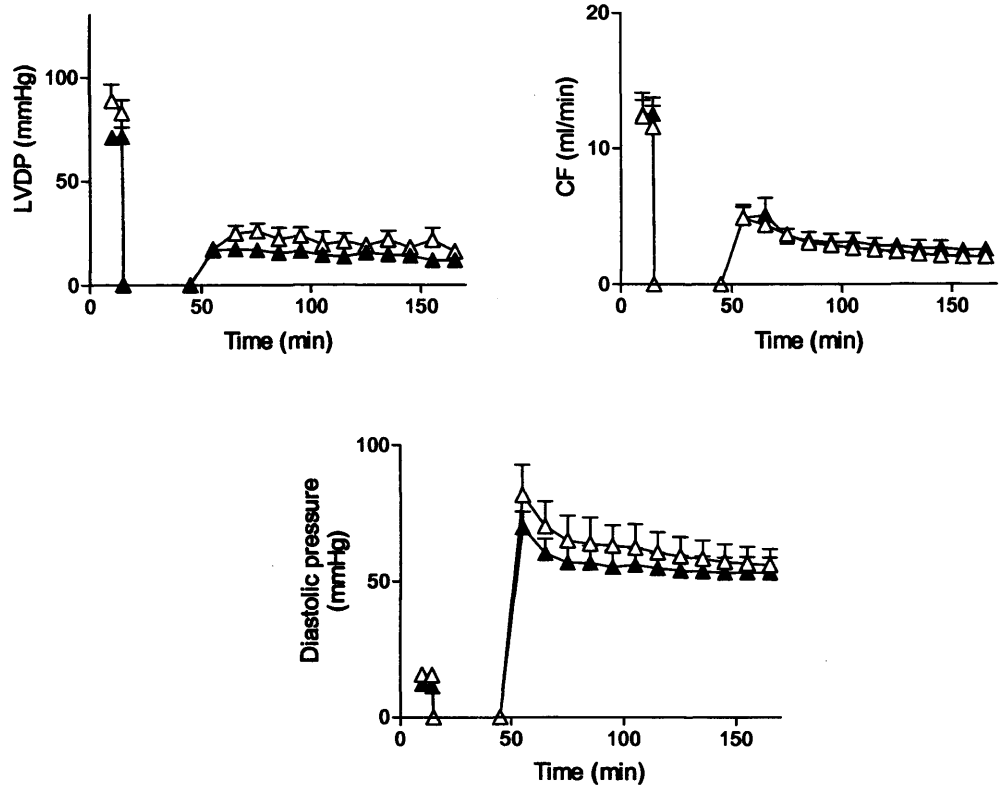


Figure 4.5 *Left ventricular developed pressure (LVDP), coronary flow (CF) and diastolic pressure for hearts treated with Tocrisolve™ 100 (n = 8) or 1μM anandamide (n = 7) in the presence of 0.01% DMSO. There were no significant differences in recoveries of coronary flow, left ventricular developed pressure or diastolic pressure among any of the factors (ANOVA with repeated measures of analysis).*



△ Tocrisolve™ 100 + SR141716A ▲ Anandamide + SR141716A

Figure 4.6 *Left ventricular developed pressure (LVDP), coronary flow (CF) and diastolic pressure for hearts treated with Tocrisolve™ 100 (n = 6) or 1μM anandamide (n = 6) in the presence of 1μM SR141716A. There were no significant differences in recoveries of coronary flow, left ventricular developed pressure or diastolic pressure among any of the factors (ANOVA with repeated measures of analysis).*

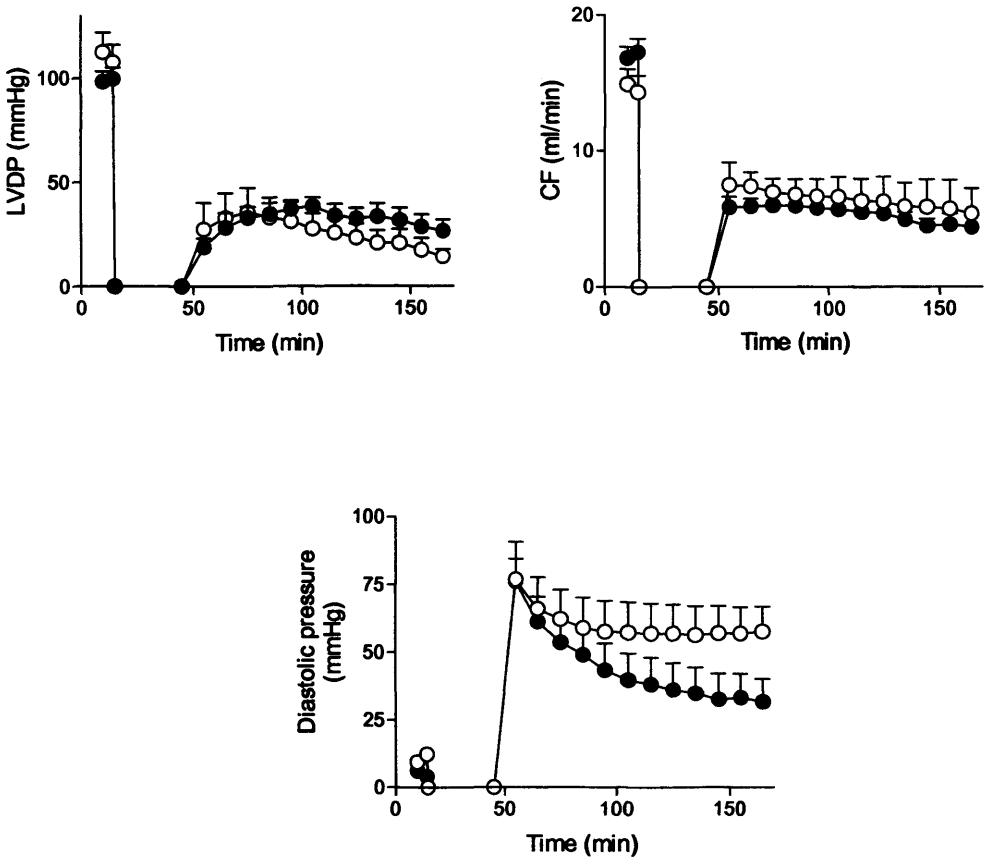


Figure 4.7 *Left ventricular developed pressure (LVDP), coronary flow (CF) and diastolic pressure for hearts treated with Tocrisolve™ 100 (n = 6) or 1μM anandamide (n = 8) in the presence of 1μM SR144528. There were no significant differences in recoveries of coronary flow, left ventricular developed pressure or diastolic pressure among any of the factors (ANOVA with repeated measures of analysis).*

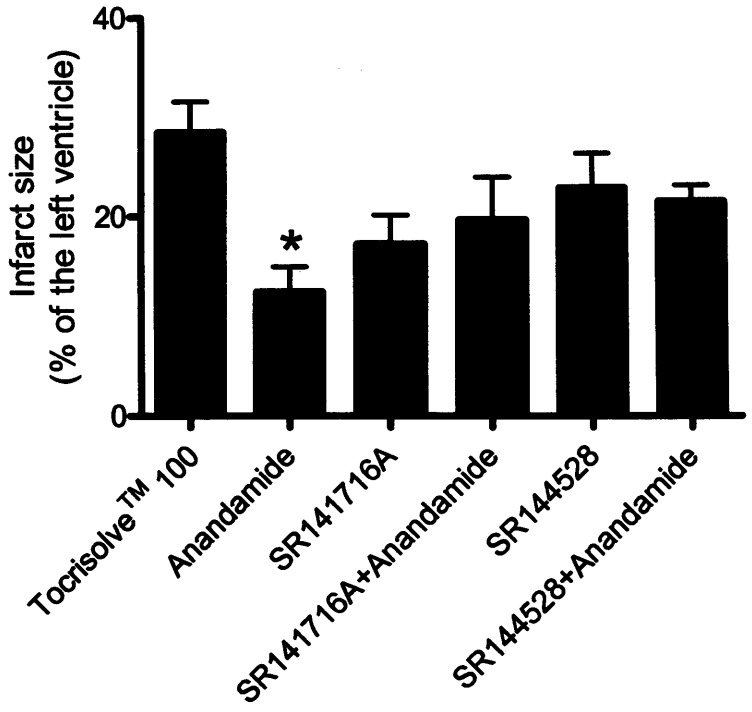


Figure 4.8 *Infarct sizes for individual hearts treated with Tocrisolve™ 100 in the absence (n = 7) and presence of 1μM SR141716A (n = 6) or 1μM SR144528 (n = 6) or 1μM anandamide in the absence (n = 7) or presence of SR141716A (n = 6) or SR144528 (n = 8) are shown. * = P<0.05 vs. control (ANOVA supported by Dunnett's post hoc test).*

4.6 Discussion

This is the first study to demonstrate that anandamide reduces infarction induced by ischaemia-reperfusion when administered prior to ischaemia in rat isolated Langendorff-buffered perfused hearts. This infarct limiting action was not mimicked by the selective cannabinoid agonists ACPA (CB_1) and JWH-133 (CB_2) either on their own or in combination. Furthermore anandamide-induced infarct size limitation was blocked in the presence of both SR141716A and SR144528, antagonists regarded as selective for CB_1 and CB_2 receptors, respectively.

The findings that anandamide-induced infarct limitation was blocked by the presence of SR141716A or SR144528 raised the possibility that stimulation of both CB_1 and CB_2 receptors or activation of one or more novel cannabinoid sites of action by anandamide was required to limit cardiac infarction associated with ischaemia-reperfusion. However, since neither ACPA nor JWH-133 alone or in combination reduced infarction activation of either CB_1 or CB_2 alone, or together, does not appear to account for cardioprotection induced by anandamide. Therefore it could be inferred that the anandamide response is a result of activation of one or more unidentified receptors.

The first indications that endocannabinoids might play a role in reducing ischaemia-reperfusion injury came from evidence concerning lipopolysaccharide-induced (Lagneux & Lamontagne, 2001) and heat stress-induced (Joyeux *et al.*, 2002) preconditioning. Both of these types of preconditioning were sensitive to SR144528 (CB_2 selective antagonist) but not to SR141716A (CB_1 selective antagonist), leading to the conclusion that the cardioprotective effect was as a result of CB_2 receptor activation.

A more recent study has examined the direct cardioprotective effects of cannabinoids (Lepicier *et al.*, 2003). In that study a low flow (0.5-0.6ml/min) model of global ischaemia was used and the endocannabinoids palmitoylethanolamide and 2-arachidonoylglycerol were shown to reduce infarction, where infarction was measured directly by infarct size and indirectly by assessing the levels of creatine kinase and lactate dehydrogenase (biochemical markers of cellular damage). Anandamide (0.3μM) a relatively non-selective endocannabinoid (Glass & Northup, 1999) was ineffective at reducing levels of creatine kinase and lactate dehydrogenase which leaked into the coronary effluent. In our study we have shown that anandamide (1μM) significantly reduced infarct size by more than 50% compared to the vehicle-treated hearts. This clearly contradicts the findings in the previous investigation. This contradiction could be the result of a number of methodological differences between the two studies. One major difference is the vehicles, which were used to deliver anandamide. Lepicier *et al.* (2003) used propylene glycol (1:9 $vol.vol^{-1}$ with Krebs-Henseleit buffer) whereas Tocrisolve™ 100 was used here. Responses to anandamide in Tocrisolve™ 100 have previously been reported (Begg *et al.*, 2002; Ford *et al.*, 2002; Kwolek *et al.*, 2005) whereas we are unaware of any reports where anandamide responses have been demonstrated with propylene glycol as the vehicle for anandamide. This is a major consideration given that responses to anandamide can be lost depending upon the solvent/vehicle used (Lopez-Miranda *et al.*, 2004).

In their study, Lepicier *et al.* (2003) found that ACEA (selective CB_1 agonist) or JWH-015 (CB_2 selective agonist), were able to reduce infarct size to a similar degree as palmitoylethanolamide and 2-arachidonoylglycerol. However, in the present study,

neither ACPA, a selective CB_1 agonist (Hillard *et al.*, 1999), nor JWH-133, a selective CB_2 receptor agonist (Huffman *et al.*, 1999), reduced infarction when used individually or in combination. At present, we have no explanation for the differences in response to CB_1 and CB_2 receptor agonists between our study and that of Lepicier *et al.* (2003). Differences in the pharmacology of the agonists used in the two are not sufficient to explain the disparities, indeed the selective agonists in our study were used at higher concentrations than those used by Lepicier *et al.* (2003). However, the difference could be due to the choice of vehicle as Lepicier *et al.* (2003) used ethanol in contrast to Tocrisolve™ 100 used in this study. Therefore, it could be argued that ACPA and JWH-133 are biologically inactive when delivered in Tocrisolve™ 100, as these preparations have not been extensively tested.

Cannabinoids are known to mediate responses via a number of different mechanisms, one of which is uptake and metabolism to form products of the arachidonic acid cascade (Ellis *et al.*, 1995; Kozak *et al.*, 2002). As methanandamide, a non-hydrolysable analogue of anandamide (Abadji *et al.*, 1994), also reduced infarct size, it is unlikely that the mechanism of cannabinoid-induced cardioprotection involves uptake, metabolism and release of arachidonic acid metabolites.

Both anandamide and methanandamide are known to activate TRPV1 (Malinowska *et al.*, 2001; Smart *et al.*, 2000). SR141716A has not been shown to block TRPV1 mediated responses (del Carmen Garcia *et al.*, 2003) therefore because SR141716A was effective in blocking the infarct limiting effect of anandamide, it is unlikely that this process is mediated via activation of TRPV1.

Cannabinoid responses can also be mediated by two receptor subtypes that have been identified and cloned, namely CB_1 (Matsuda *et al.*, 1990) and the CB_2 receptor (Munro *et al.*, 1993). Anandamide can bind and activate both of these receptors (Vogel *et al.*, 1993; Zoratti *et al.*, 2003) although there are also examples where anandamide has not activated CB_2 receptors (Bayewitch *et al.*, 1995; Facci *et al.*, 1995). Methanandamide, on the other hand, is a relatively selective agonist for the CB_1 receptor (Mechoulam *et al.*, 1998). Therefore it would appear that the infarct limiting actions of anandamide and methanandamide are as a result of the activation of the CB_1 receptor. However, the selective CB_2 antagonist, SR144528 as well as SR141716A (selective CB_1 antagonist) blocked the infarct-limiting response of anandamide. There are two explanations for this, firstly the infarct limiting action of anandamide requires both CB_1 and CB_2 receptors to be co-stimulated so that if one was blocked the cardioprotective response would be lost and secondly that the response to anandamide is mediated by a receptor distinct from either CB_1 or CB_2 but which is sensitive to the two cannabinoid antagonists.

We found that individually neither the selective CB_1 agonist ACPA (Hillard *et al.*, 1999) nor the selective CB_2 agonist JHW-133 (Pertwee, 1999) reduced infarct size. This would be expected if co-stimulation of the receptors was required. However, when the agonists were used in combination, the size of infarction should have been reduced but our data demonstrates that this is not the case suggesting that the anandamide-induced cardioprotection is not mediated by synergistic activation of CB_1 and CB_2 receptors, favouring the theory that the response is mediated at a site distinct from these receptor subtypes.

The presence of a cannabinoid receptor distinct from either the CB_1 or CB_2 receptor located on endothelial cells has recently been proposed (Mo *et al.*, 2004). Anandamide is thought to be able to activate this receptor therefore activation of the putative endothelial cannabinoid receptor (Jarai *et al.*, 1999) may play a role in the mechanism of anandamide infarct size limitation. However, SR144528 abolished the response to anandamide which indicates that the putative endothelial cannabinoid receptor is not involved as it is reportedly insensitive to this antagonist (Mo *et al.*, 2004).

It has previously been reported that anandamide mediates cardiac responses such as negative inotropy and coronary vasodilation through interaction with a novel site distinct from any of the known cannabinoid pathways (Ford *et al.*, 2002). The pharmacological profile of the anandamide infarct limiting effect seen in this study is the same as that previously reported (Ford *et al.*, 2002). Therefore, it would appear that in addition to that study anandamide reduces infarct size by interaction with one or more novel mechanisms of cannabinoid signal transduction similar to those, which mediate negative inotropy and coronary vasodilation.

In summary, we have shown that anandamide and its stable analogue methanandamide limit infarct size associated with myocardial ischaemia-reperfusion without affecting contractile recovery. The pharmacological profile of this response is the same as that previously reported for anandamide-induced cardiac responses (Ford *et al.*, 2002) and fails to match with any of the previously known mechanisms of cannabinoid action. We therefore conclude that anandamide reduces infarct size in rat isolated hearts by

interaction with one or more novel sites of action that might involve a new cannabinoid receptor subtype.

Chapter 5

Investigation into the role of endocannabinoids on ischaemia-reperfusion injury induced in a regional ischaemic model

Table of Contents

5.1 Introduction

In the previous chapter, hearts subjected to global no-flow ischaemia were used to test whether cannabinoids protect the myocardium from ischaemia-reperfusion injury. However, whole heart ischaemia is not a common clinical occurrence mainly being limited to cardiac transplantation. The most common clinical manifestation of cardiac ischaemia occurs during myocardial infarction where an occlusion, caused when a ruptured atherosclerotic plaque, blocks a coronary artery causing the area downstream from the plaque to become ischaemic. During a myocardial infarction the whole heart would not be affected, instead a smaller area would be subjected to ischaemia-reperfusion injury.

Another limitation of a global ischaemia model is that it cannot be guaranteed that drug treatment will reach all areas of a heart, which has been subjected to global ischaemia, whereas in a regionally ischaemic heart the area is smaller and therefore there is a greater likelihood that the drug will reach all the areas that have been damaged. In the global model the drug is applied before ischaemia and therefore it could also be argued that the results seen are due to preconditioning. Instead the drug could be introduced at reperfusion however, coronary flow is a limiting factor and therefore drug penetration may be compromised. For these reasons a regional model of cardiac ischaemia was chosen to better model the clinical situation.

The current clinical treatment for patients suffering occlusion is either thrombolytics or coronary artery angioplasty. Thrombolytics should be given as soon as possible (preferably in the first two hours after the onset of symptoms) so as to increase

myocardial salvage (Department of Health, 2000). It would be beneficial to introduce a drug at this stage which would not interfere with the restoration of flow but would help to limit the subsequent damage caused by the onset of reperfusion.

The regional ischaemia model does have its own drawbacks in that for a myocardial infarction to occur in a clinical situation the patient would have other pathophysiological features for example atherosclerosis, which are not present in this method as hearts have been harvested from rats without any apparent cardiovascular disease.

Regional ischaemia models are widely used to assess cardioprotection. This model has previously been used to investigate ischaemia-reperfusion with examples as diverse as insulin-mediated cardioprotection (Jonassen *et al.*, 2001), to assess at what time point adenosine would need to be present at reperfusion for it to be cardioprotective (Xu *et al.*, 2003) and to assess if preconditioning can limit infarct size in a mouse model (Miller & Van Winkle, 1999). At present there have been no investigations into the cardioprotective effects of cannabinoids using this model.

5.2 Experimental protocol

A constant pressure Langendorff buffered-perfused heart preparation was used with hearts undergoing a period of regional ischaemia. A detailed description of the methodology can be found in section 2. 3. In brief, following the attachment of the aorta to the cannula and immersion of the heart into an organ bath, hearts were aerobically perfused for the first 15 minutes (baseline values taken at 10 minutes and pre-ischaemic values at 15 minutes) before undergoing a 40 minute period of ischaemia induced by tying a snare around the left anterior descending coronary artery (Figure 5.1 shows the position of the left anterior descending coronary artery and where the snare was placed).

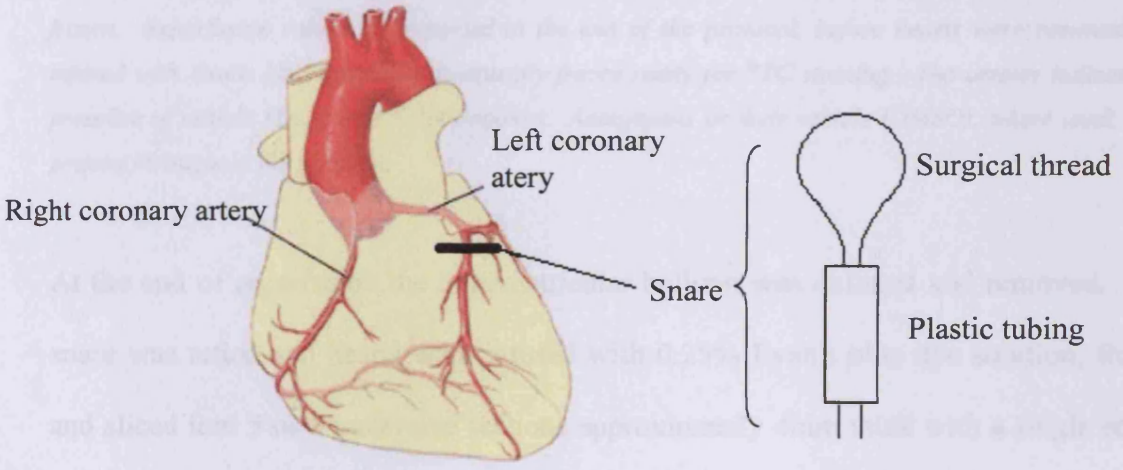


Figure 5.1 *Diagram of the heart showing the position of the snare. Regional ischaemia is induced by ligating the left anterior descending coronary artery. To prevent tearing of the heart tissue and also to facilitate reperfusion, the occluding ligature is tied against a small length of plastic tubing. Reperfusion is induced by releasing the snare.*

Agonists were infused 35 minutes into ischaemia with the infusion continuing throughout the subsequent 1 hour of reperfusion. Reperfusion was defined as occurring

once the snare around the left anterior descending coronary artery was released. Figure 5.2 shows the experimental protocol used for these experiments.

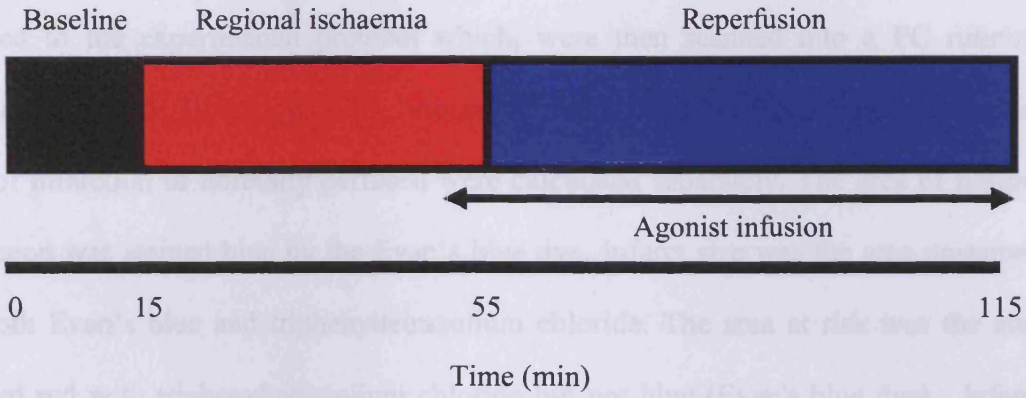


Figure 5.2 *Diagrammatic representation of the regional ischaemia protocol. Hearts were aerobically perfused for the first 15 minutes (black). Baseline values were taken at 10 minutes and pre-ischaemic values were taken just before ischaemia was induced. Reperfusion commenced after 40 minutes of ischaemia (red). Hearts were reperfused for 1 hour (blue) and then frozen. Reperfusion values are reported at the end of the protocol, before hearts were removed and infused with Evans blue dye and subsequently frozen ready for TTC staining. The arrows indicate the presence of vehicle (Tocrisolve™ 100)/agonist. Antagonists or their vehicle (DMSO), where used, were present throughout the protocol.*

At the end of reperfusion the intraventricular balloon was deflated and removed. The snare was retied and hearts were infused with 0.25% Evan's blue dye solution, frozen and sliced into 5 or 6 transverse sections approximately 4mm thick with a single edged razor. Areas of connective tissue were removed during this process. The slices were then placed in a beaker containing a 1% solution of triphenyltetrazolium chloride for 5-10 minutes until a brick red colour had developed. Stained hearts were stored in a 10% formalin solution (formaldehyde solution, phosphate buffered, pH 7.3) for 24 hours. The stained slices were flattened between two glass plates separated by 2.5mm. Areas stained blue by the Evan's blue stain indicated the area which was not at risk of

infarction, the presence of a red stain (triphenyltetrazolium chloride) indicated the viable cells within the area at risk of infarction and the absence of any staining indicated areas of infarction. These areas were traced onto an acetate sheet by an investigator blinded to the experimental protocol which, were then scanned into a PC running SigmaScan Pro 5 (Hounslow, UK). Areas (in pixels) identified as being infarcted, at risk of infarction or normally perfused were calculated separately. The area of normal perfusion was stained blue by the Evan's blue dye. Infarct size was the area unstained by both Evan's blue and triphenyltetrazolium chloride. The area at risk was the area stained red with triphenyltetrazolium chloride but not blue (Evan's blue dye). Infarct size is expressed as a percentage of the area at risk. Figure 5.3 shows a control heart stained in this manner.

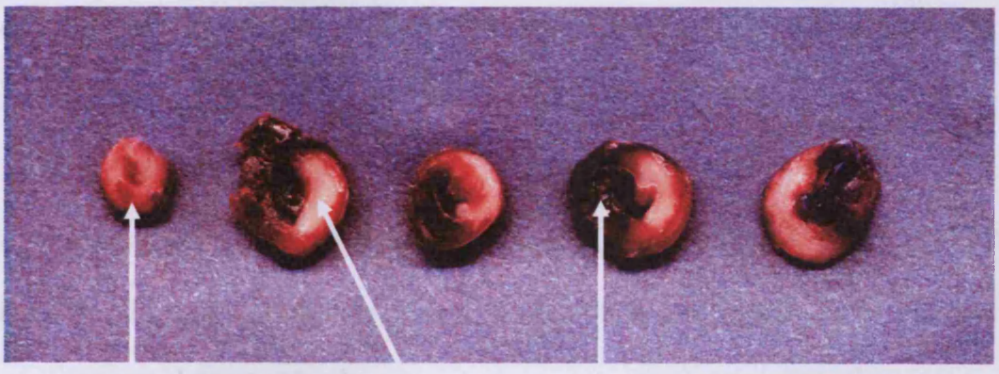


Figure 5.3 *Picture showing a typical example of a stained heart.*

5.21 Agonist Studies

Hearts were randomly assigned to receive the cannabinoid vehicle, Tocrisolve™ 100 (a vehicle of 1:4 soya oil:water mixture emulsified with poloxamer F188), or one of the

following cannabinoid agonists (1μM): anandamide, methanandamide, arachidonlycyclopropylamide (ACPA), JWH-133 or a combination of ACPA and JWH-133 (1μM each). Cannabinoid agonists or Tocrisolve™ 100 vehicle were infused at a flow rate controlled to be 10% of coronary flow.

5.22 Antagonist studies

Where used, antagonists were present in the perfusate throughout the protocol. Antagonists were prepared in dimethyl sulfoxide (DMSO) as a 1mM stock solution before being added to the perfusate (final concentration 1μM).

Hearts were randomly assigned to one of four groups. The first consisted of the antagonist vehicle DMSO (0.01% vol.vol^{-1}). The second group was treated with SR141716A the third group with SR144528 and the fourth group with capsazepine.

5.3 Drug preparation

The cannabinoid agonists, anandamide, methanandamide, ACPA, and JWH-133 (Tocris Cookson Ltd, Bristol, UK) obtained as solutions of 5mg/ml dissolved in Tocrisolve™ 100 were diluted in Krebs bicarbonate solution to a concentration of 10^{-5}M so that when infused at a rate controlled to be 10% of coronary flow a final concentration of 1μM was obtained. The cannabinoid agonist vehicle Tocrisolve™ 100 was diluted in Krebs bicarbonate to give a 0.07% vol.vol^{-1} solution that when infused at 10% of coronary flow a final concentration of 0.007% vol.vol^{-1} was obtained. SR141617A and SR144528 (gifts from Dr Barth of Sanof-Aventis, Montpellier, France) and capsazepine

(Sigma, Gillingham, UK) were prepared in DMSO (Fisher Scientific, Leicestershire, UK) as 1mM stock solutions and added to the perfusate (final concentration was 1μM).

5. 4 Statistical analysis

All data are expressed as the mean ± SEM of *n* experiments. Unpaired data were compared using an unpaired Student's t-test. Multiple comparisons against a single control were made using analysis of variance (ANOVA) followed by Dunnet's *post hoc* test (GraphPad InStat 3.0, California, USA). ANOVA with repeated measures was used to compare time course data (SPSS Inc, Illinois, USA). Statistical significance was taken at $P < 0.05$.

5.5 Results

5.51 Agonists Studies

Left ventricular pressure, rate pressure product, heart rate and coronary flow did not significantly vary among any of the agonist treated groups before ischaemia (Table 5.1).

In vehicle treated (Tocrisolve™ 100) control hearts, left ventricular pressure recovered incompletely during reperfusion recovering to a maximum of 90 ± 4% of pre-ischaemia values after 10 minutes of reperfusion. Thereafter left ventricular developed pressure steadily declined until the end of reperfusion such that by the end of reperfusion the values were 51 ± 4% of pre-ischaemic values (Figure 5.4a and Table 5.1). Recovery of coronary flow during reperfusion was also incomplete reaching a maximum (77 ± 7% of pre-ischaemic values) after 10 minutes of reperfusion, declining to 43 ± 7% of pre-ischaemic values by the end of reperfusion (Figure 5.4a and Table 5.1). Heart rate declined steadily over the experimental period to 79 ± 6% of pre-ischaemic values (Figure 5.4b and Table 5.1). Recovery of rate pressure product was also incomplete reaching a maximum of 78 ± 7% of pre-ischaemic values after 10 minutes of reperfusion, then declining to 41 ± 5% of pre-ischaemic values by the end of reperfusion (Figure 5.4b and Table 5.1). The area at risk was 30 ± 1% of the whole heart with infarct size, measured at the end of reperfusion at 28 ± 3% of the area at risk (Figure 5.5 and Table 5.1).

None of the cannabinoid agonists used in the study had a significant effect on the recoveries of left ventricular pressure, rate pressure product or coronary flow compared to control hearts (Figures 5.4a and 5.4b and Table 5.1). However, infarct size was

significantly reduced by the presence of methanandamide (10 ± 5% of the area at risk where the area at risk was 32 ± 3% of the whole heart) compared to hearts which received vehicle. Infarct sizes in hearts treated with anandamide (23 ± 6% of the area at risk where the area at risk was 31 ± 2% of the whole heart, Figure 5.5), ACPA (35 ± 3% of the area at risk, where the area at risk was 27 ± 3% of the whole heart) or JWH-133 (24 ± 3% of the area at risk, where the area at risk was 30 ± 2% of the whole heart) individually or in combination were not significantly different from those obtained in vehicle control hearts (Figure 5.5 and Table 5.1). However ACPA and JWH-133 in combination were significantly different from those obtained in vehicle control hearts (51 ± 4% of the area at risk where the area at risk was 23 ± 1% of the whole heart; Figure 5.5 and Table 5.1).

5.52 Antagonist studies

None of the antagonists used had a significant effect on left ventricular pressure, coronary flow, heart rate or rate pressure product before ischaemia (Table 5.2).

In vehicle treated control hearts with 0.01% vol.vol^{-1} DMSO (antagonist vehicle) present, recovery of left ventricular pressure was incomplete reaching a maximum of 75 ± 6% of pre-ischaemic values after 10 minutes of reperfusion then falling to 42 ± 6% of pre-ischaemic values by the end of reperfusion (Figure 5.6 and Table 5.2). Coronary flow was impaired during reperfusion recovering to a maximum of 56 ± 7% of pre-ischaemic values after 10 minutes of reperfusion then falling to 33 ± 8% of pre-ischaemic values by the end of reperfusion (Figure 5.6 and Table 5.2). Rate pressure product recovered incompletely during reperfusion recovering to a maximum of 59 ±

7% of pre-ischaemic values after 10 minutes of reperfusion, then declining until at the end of reperfusion the values were 29 ± 6% of pre-ischaemic values (Figure 5.6 and Table 5.2). Measured at the end of reperfusion, 30 ± 6% of the area at risk was infarcted where the area at risk was 33 ± 5% of the whole heart (Figure 5.6 and Table 5.2).

Infusion of methanandamide in the presence of 0.01% $vol.vol^{-1}$ DMSO had no significant effect on the recovery of left ventricular pressure, rate pressure product or coronary flow during reperfusion compared to vehicle control hearts (Figure 5.6 and Table 5.2). However infarct size, measured at the end of reperfusion, was significantly reduced to 11 ± 3% of the area at risk where the area at risk was 48 ± 5% of the whole heart (Figure 5.6 and Table 5.2).

No parameters of contractile function, area at risk or infarct size significantly varied between vehicle-treated hearts in the presence of 0.01% $vol.vol^{-1}$ DMSO compared to hearts treated with SR141716A (1μM, Figure 5.7), SR144528 (1μM, Figure 5.8) or capsazepine (1μM, Figure 5.9).

Recoveries of left ventricular developed pressure, rate pressure product and coronary flow during reperfusion in hearts treated with methanandamide were not significantly affected by the presence of SR141617A, SR144528 or capsazepine when compared to SR141617A, SR144528 or capsazepine alone (Figures 5.7-5.9 and Table 5.2). However, the infarct size reduction observed when methanandamide was infused alone

was lost in the presence of SR141617A and SR144528 but not in the presence of capsazepine (Figure 5.9).

Table 5.1 *Cardiac function and area at risk of infarction in hearts treated with cannabinoid agonists. Times correspond to pre-drug (10 min), pre-ischaemic (15 min) and the time at the end of reperfusion (115 minutes).*

	Perfusion Time (min)	*n*	LVDP (mmHg)	CF ($ml.min^{-1}$)	BPM	% AAR
Tocrisolve™ 100	10	8	109±10	12±1	287±12	30±1
	15		105±8	11±1	272±14	
	115		52±4	5±1	217±22	
Anandamide (1μM)	10	9	102±4	14±1	255±9	31±2
	15		104±4	14±1	246±8	
	115		61±5	5±1	163±21	
Methanandamide (1μM)	10	7	105±12	13±1	245±8	32±3
	15		106±13	12±1	238±12	
	115		46±9	4±1	110±45	
ACPA (1μM)	10	7	117±8	13±1	252±10	27±3
	15		118±7	13±1	243±13	
	115		60±13	6±1	191±8	
JWH-133 (1μM)	10	7	97±11	11±1	259±8	30±2
	15		99±11	10±1	248±5	
	115		75±9	4±0	208±11	
ACPA + JWH-133 (1μM each)	10	8	105±10	11±1	263±12	23±1
	15		107±11	11±1	251±12	
	115		72±9	6±1	182±11	

LVDP = left ventricular developed pressure, CF = coronary flow and BPM = beats per minute. Absolute values are given as mean ± s.e.m. with *n* the number of hearts in the group.

Table 5.2 *Cardiac function and area at risk of infarction in hearts treated with cannabinoid agonists and in the presence of the cannabinoid antagonists. Times correspond to pre-drug (10 min), pre-ischaemic (15 min) and the time at the end of reperfusion (115 minutes).*

	Perfusion Time (min)	*n*	LVDP (mmHg)	CF ($ml.min^{-1}$)	BPM	% AAR
Tocrisolve™ 100	10 15 115	6	101±10 99±12 44±11	14±1 14±1 5±1	266±6 260±6 177±13	33±5
mAEA (1μM)	10 15 115	7	110±8 103±8 37±5	15±1 14±1 3±2	267±15 261±15 114±25	48±5
SR141716A (1μM)	10 15 115	7	90±12 93±12 57±7	13±1 12±1 6±1	256±13 248±13 175±13	29±3
SR141716A + mAEA (1μM)	10 15 115	10	107±7 112±8 56±10	11±1 11±1 5±1	259±11 241±10 172±19	27±3
SR144528 (1μM)	10 15 115	6	94±16 92±15 48±13	14±1 14±1 7±1	278±12 278±11 196±25	29±4
SR144528 + mAEA (1μM)	10 15 115	6	118±14 118±13 69±8	14±1 13±1 5±1	274±10 271±13 141±28	30±2
Capsazepine (1μM)	10 15 115	7	124±13 120±14 54±12	14±1 14±1 5±1	273±7 258±6 194±19	30±2
Capsazepine + mAEA (1μM)	10 15 115	7	112±13 110±13 47±8	15±2 15±2 5±2	288±12 275±13 140±25	27±1

LVDP = left ventricular developed pressure, CF = coronary flow, BPM = beats per minute and mAEA = methanandamide. Absolute values are given as mean ± s.e.m. with *n* the number of hearts in the group.

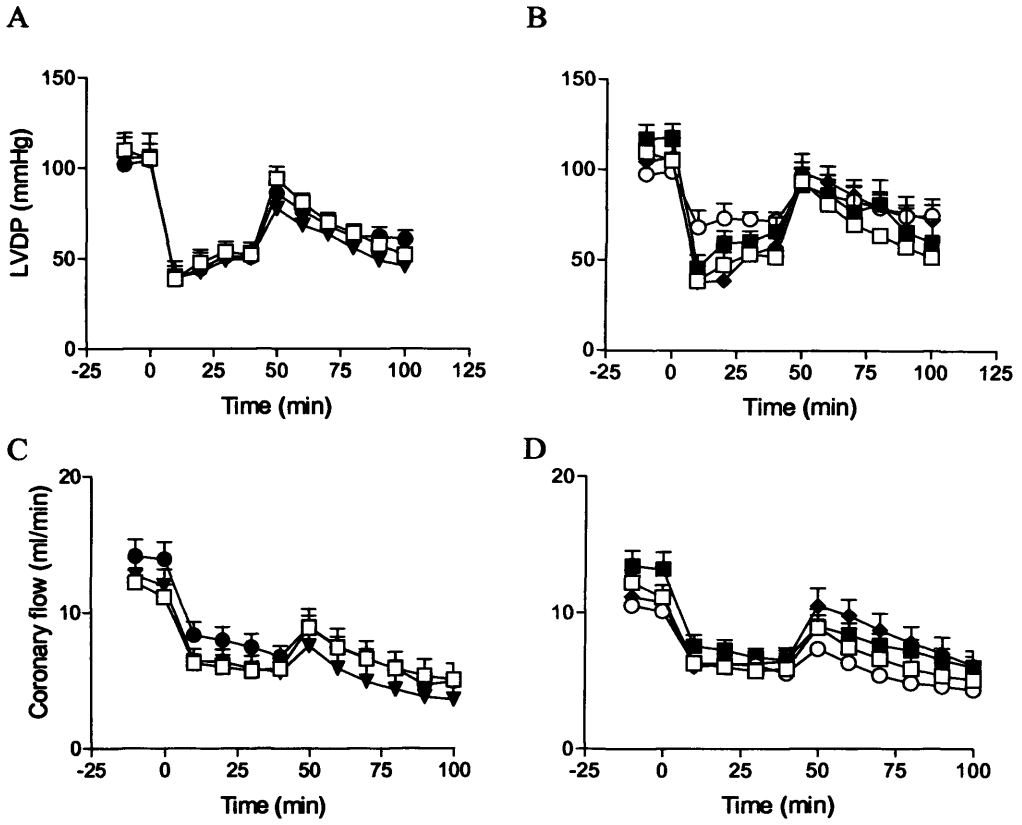


Figure 5.4a *Left ventricular developed pressure (LVDP, panels A and B) and coronary flow (CF, panels C and D) for hearts treated with Tocrisolve™ 100 (□, n = 8) or 1μM anandamide (●, n = 9), 1μM methanandamide (▼, n = 7), 1μM ACPA (■, n = 8) ,1μM JWH-133 (○, n = 7), and a combination of 1μM ACPA and 1μM JWH-133 (◆, n = 8) . To aid comparison the same control group appears in all panels. LVDP, RPP and CF did not significantly vary among any of the experimental groups (ANOVA with repeated measures of analysis).*

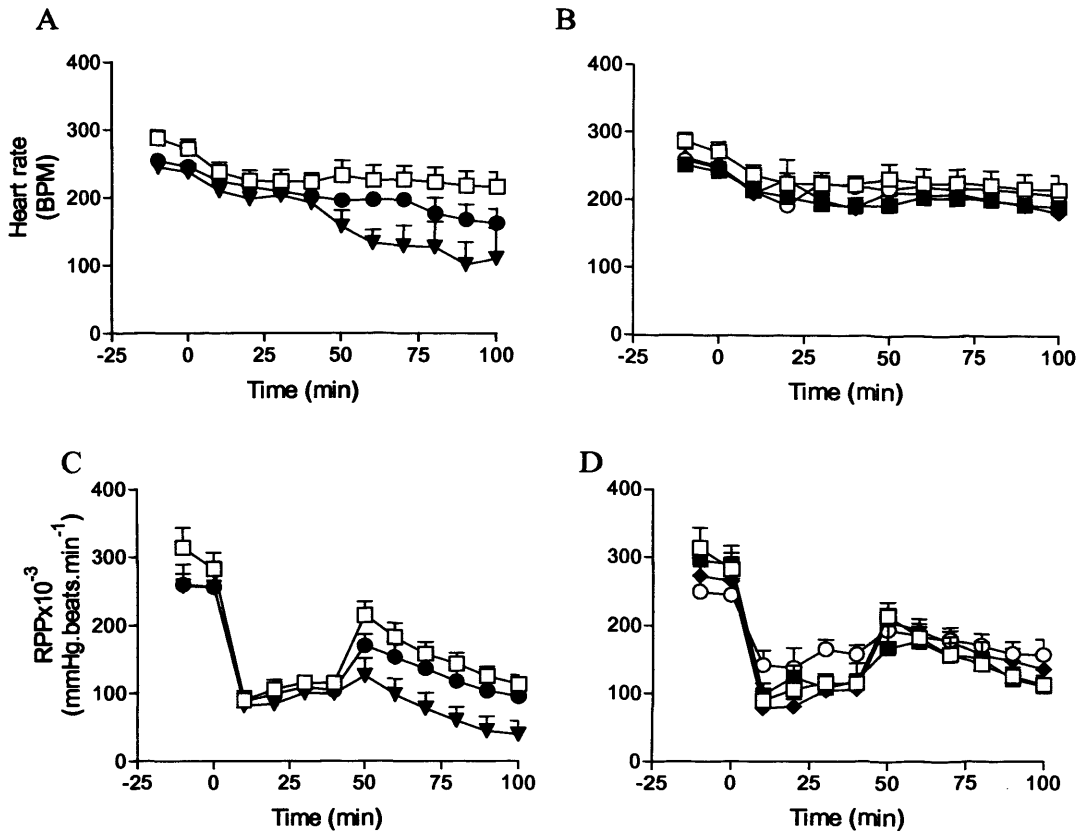


Figure 5.4b *Heart rate (BPM, panels A and B) and rate pressure product (RPP, panels C and D) for hearts treated with Tocrisolve™ 100 (□, n = 13) or 1μM anandamide (●, n = 7), 1μM methanandamide (▼, n = 6), 1μM ACPA (■, n = 6) ,1μM JWH-133 (○, n = 8), and a combination of 1μM ACPA and 1μM JWH-133 (◆, n = 9) . To aid comparison the same control group appears in all panels. LVDP, RPP and CF did not significantly vary among any of the experimental groups (ANOVA with repeated measures of analysis).*

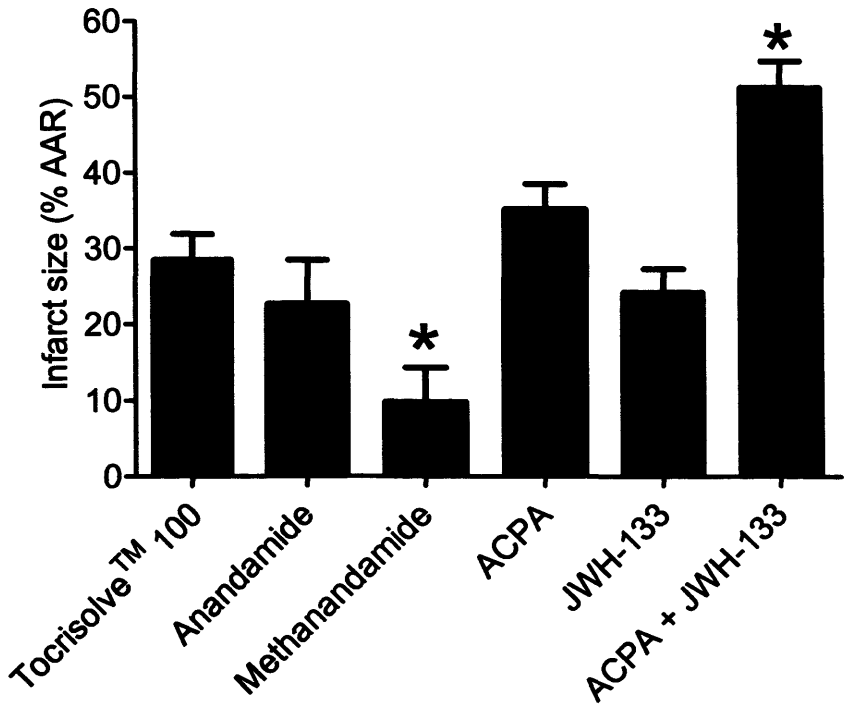


Figure 5.5 *Infarct sizes for hearts treated with Tocrisolve™ 100 (n = 8) or 1 μM anandamide (n = 9), 1 μM methanandamide (n = 7), 1 μM ACPA (n = 7), 1 μM JWH-133 (n = 7) and a combination of 1 μM ACPA and 1 μM JWH-133 (n = 10). * = P<0.05 vs. vehicle (ANOVA supported by Dunnett's post hoc test).*

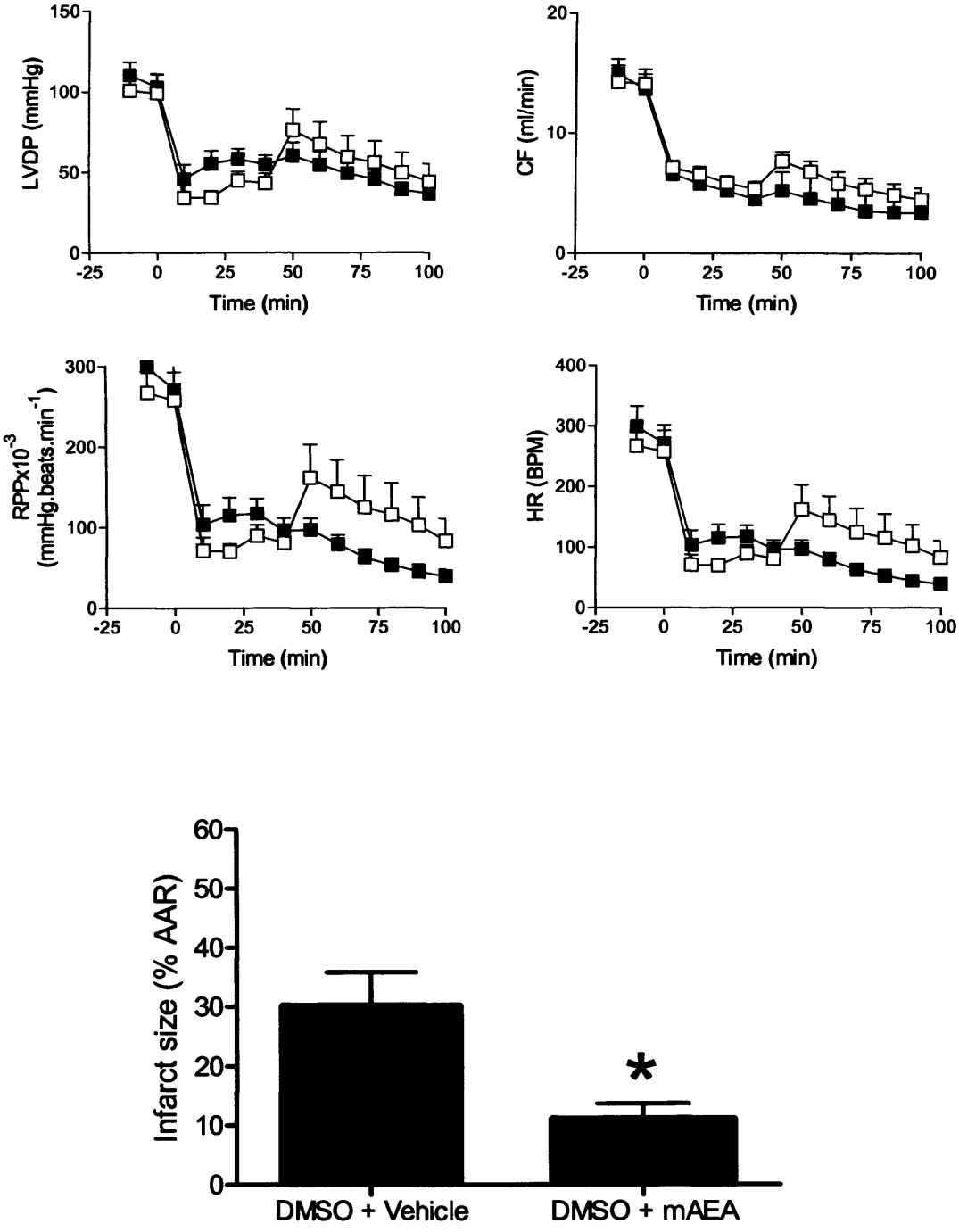


Figure 5.6 *Left ventricular developed pressure (LVDP), coronary flow (CF), rate pressure product (RPP), heart rate (HR) and infarct size for hearts treated with Tocrisolve™ 100 (vehicle, □, n = 6) or 1μM methanandamide (mAEA, ■, n = 7) in the presence of 0.01% DMSO. * = P<0.05 vs. vehicle (Student's t-test was used to analyse the data).*

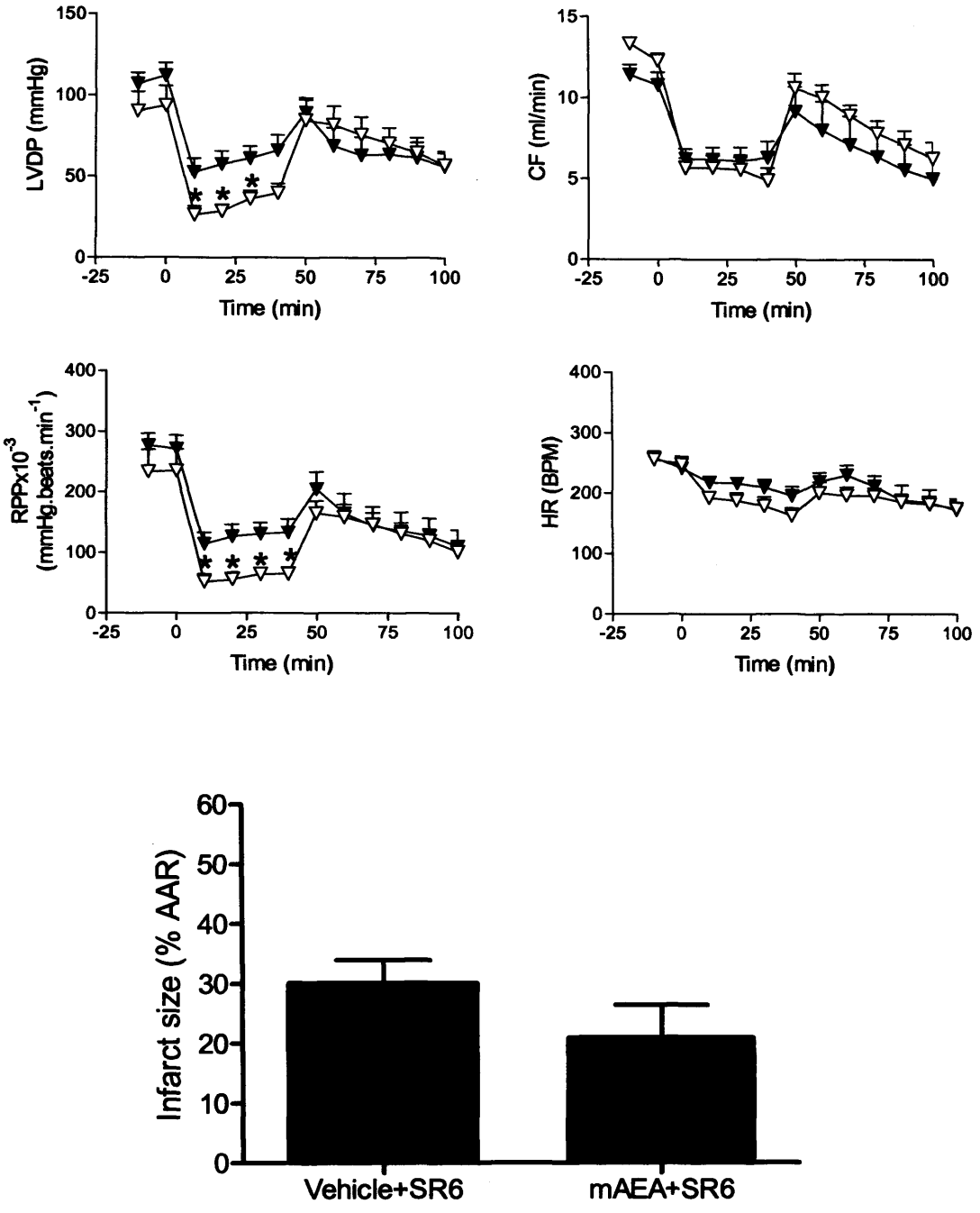
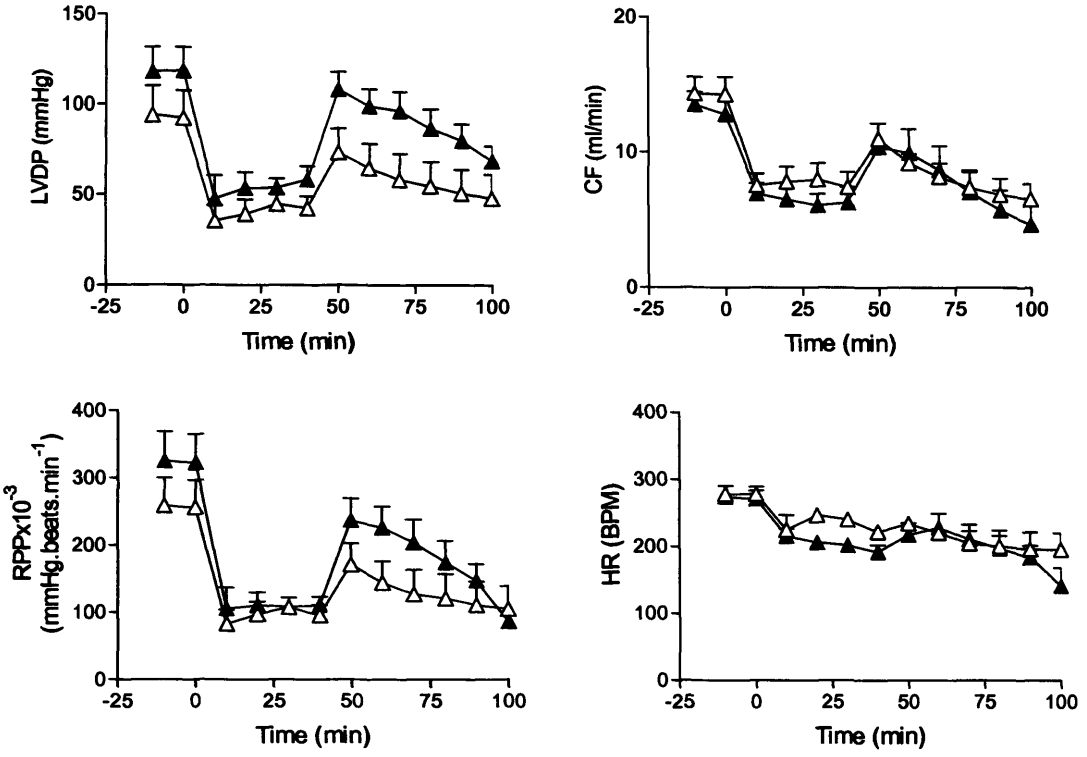


Figure 5.7 *Left ventricular developed pressure (LVDP), coronary flow (CF), rate pressure product (RPP), heart rate (HR) and infarct size for hearts treated with Tocrisolve™ 100 (vehicle, ▽, n = 7) or 1μM methanandamide (mAEA, ▼, n = 10) in the presence of 1μM SR141716A (SR6). There were no significant differences in recoveries of coronary flow, left ventricular developed pressure, rate pressure product and heart rate among any of the factors or in infarct size. * = P<0.05 vs. vehicle (Student's t-test was used to analyse the data).*



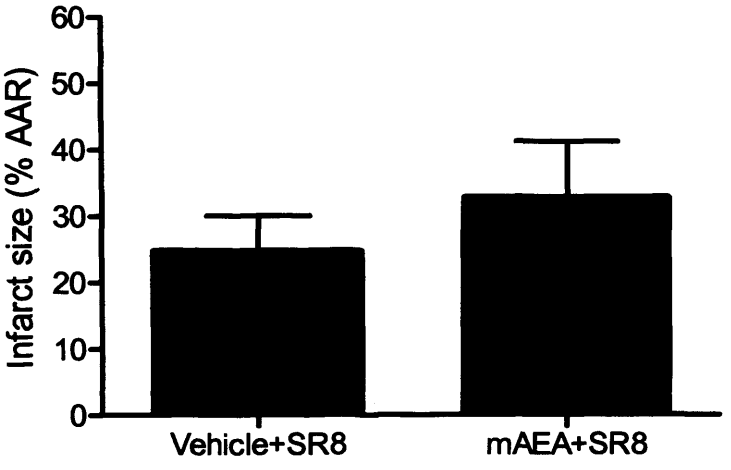


Figure 5.8 *Left ventricular developed pressure (LVDP), coronary flow (CF), rate pressure product (RPP), heart rate (HR) and infarct size for hearts treated with Tocrisolve™ 100 (vehicle, △, n = 6) or 1μM methanandamide (mAEA, ▲, n = 6) in the presence of 1μM SR144528 (SR8). There were no significant differences in recoveries of coronary flow, left ventricular developed pressure, rate pressure product and heart rate among any of the factors or in infarct size (Student's t-test was used to analyse the data).*

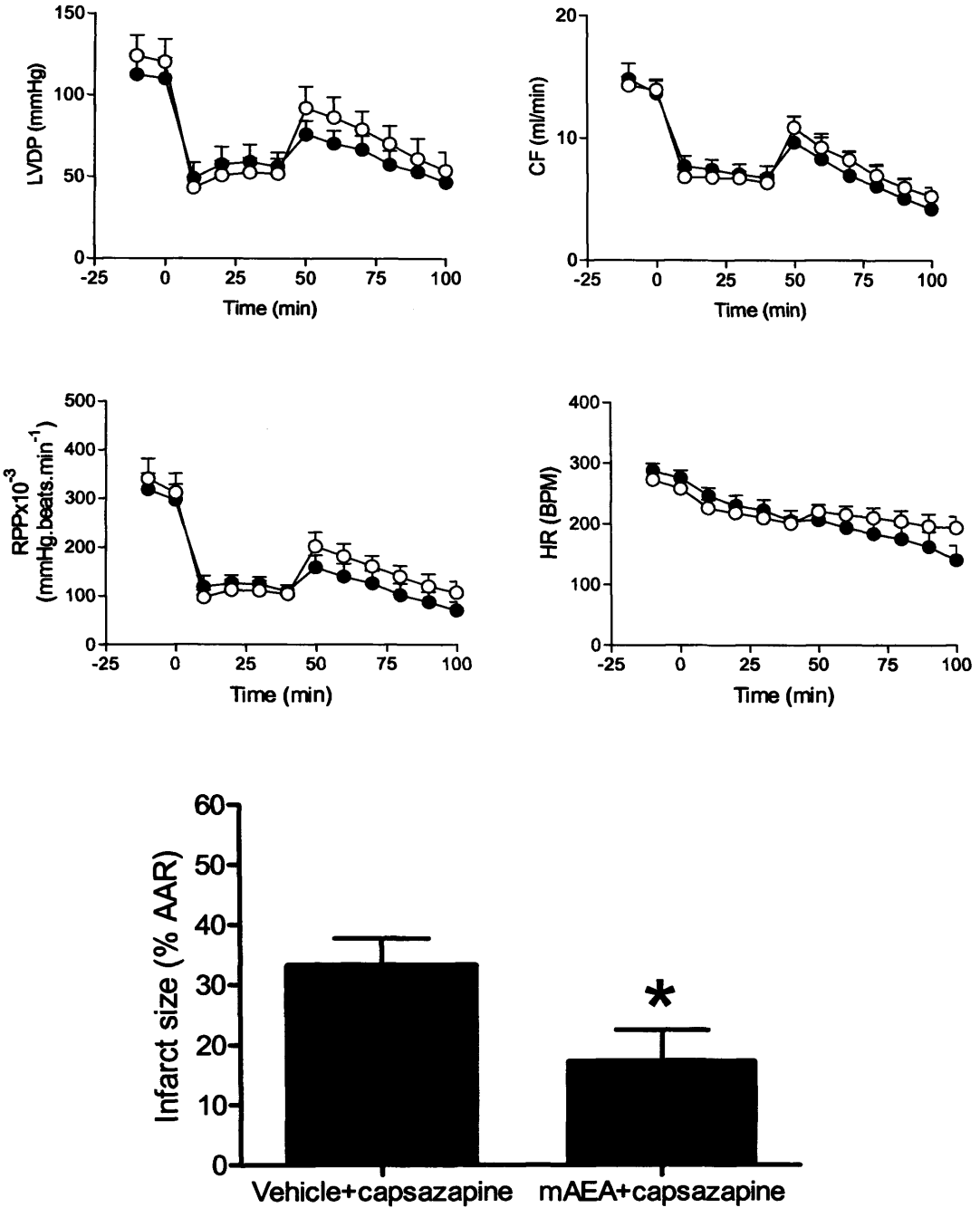


Figure 5.9 *Left ventricular developed pressure (LVDP), coronary flow (CF), rate pressure product (RPP), heart rate (HR) and infarct size for hearts treated with Tocrisolve™ 100 (vehicle, ○, n = 7) or 1μM methanandamide (mAEA, ●, n = 7) in the presence of in the presence of 1μM capsazepine.* * = *P<0.05 vs. vehicle (Student's t-test was used to analyse the data).*

5.6 Discussion

This is the first study to demonstrate that cannabinoids can reduce infarct size when delivered at a clinically relevant time point. Although previous studies have demonstrated that cannabinoids are cardioprotective (Joyeux *et al.*, 2002; Lagneux & Lamontagne, 2001; Lepicier *et al.*, 2003), this is the first where cannabinoids administered after the onset of ischaemia have been shown to reduce infarct size. This is important as it demonstrates that the mechanism of protection is independent of preconditioning.

The main difference between this and the previous findings in Chapter 4 is that anandamide did not reduce infarct size. The major difference between anandamide and methanandamide is that methanandamide is non-hydrolysable and therefore is not broken down when taken up into cells. When anandamide is broken down the diffusion gradient by which anandamide is taken up into the cell does not slow down, however, methanandamide is not broken down and therefore the concentration gradient is overcome and consequently methanandamide is left in contact with the tissue for a longer period of time than anandamide allowing it time to protect the tissue from damage.

There are two cannabinoid receptors that have been identified and cloned, namely CB_1 (Matsuda *et al.*, 1990) and the CB_2 receptor (Munro *et al.*, 1993). Methanandamide, is a relatively selective agonist for the CB_1 receptor (Mechoulam *et al.*, 1998). Therefore it would appear that the infarct limiting actions of methanandamide are as a result of the activation of the CB_1 receptor. However, the selective CB_2 antagonist, SR144528 as

well as SR141716A (selective CB_1 antagonist) blocked the infarct-limiting response to methanandamide. There are two explanations for this, firstly the infarct limiting action of methanandamide requires both CB_1 and CB_2 receptors to be co-stimulated so that if one was blocked the cardioprotective response would be lost and secondly that the response to methanandamide is mediated by a receptor distinct form either CB_1 or CB_2 but which is sensitive to the two cannabinoid antagonists.

It could be inferred that the methanandamide response is a result of activation of one or more unidentified receptors. As with the previous chapter (Chapter 4), the findings that methanandamide-induced infarct limitation was blocked by the presence of SR141617A or SR144528 raised the possibility that co-stimulation of the CB_1 and CB_2 receptors or activation of one or more novel cannabinoid sites of action by methanandamide is required to limit cardiac infarction associated with ischaemia-reperfusion. However, since neither the CB_1 receptor agonist, ACPA (Hillard *et al.*, 1999) or the CB_2 receptor agonist, JWH-133 (Pertwee, 1999) either on their own or in combination reduced infarction, activation of either CB_1 or CB_2 alone, or simultaneously, does not appear to account for the cardioprotection afforded by methanandamide favouring the theory that the response is mediated at a site distinct from these receptor subtypes.

Cannabinoids are known to mediate responses via a number of different mechanisms, one of which is uptake and metabolism to form products of the arachidonic acid cascade (Ellis *et al.*, 1995; Kozak *et al.*, 2002). As methanandamide is a non-hydrolysable analogue of anandamide (Abadji *et al.*, 1994) responses seen to methanandamide are not

due to release of arachidonic acid metabolites formed due to the breakdown of this cannabinoid.

Methanandamide is known to activate TRPV1 (Malinowska *et al.*, 2001). Therefore, it is possible that TRPV1 activation might be involved in the mechanism of cardioprotection. However, capsazepine (TRPV1 antagonist) did not block the infarct limiting effect of methanandamide, therefore it is unlikely that this process is mediated via activation of TRPV1.

The presence of a cannabinoid receptor distinct from either the CB_1 or CB_2 receptor located on endothelial cells has recently been proposed (Mo *et al.*, 2004). Anandamide is thought to be able to activate this receptor therefore activation of the putative endothelial cannabinoid receptor (Jarai *et al.*, 1999) may play a role in the mechanism of methanandamide infarct size limitation due to their similarities. However, SR144528 abolished the response to methanandamide which indicates that the putative endothelial cannabinoid receptor is not involved as it is reportedly insensitive to this antagonist (Mo *et al.*, 2004).

In summary, we have shown that methanandamide limits infarct size associated with myocardial ischaemia-reperfusion injury without affecting contractile recovery. The pharmacological profile of this response is the same as that previously reported for the cardiac responses to methanandamide's parent compound anandamide (Ford *et al.*, 2002) and fails to match with any of the previously known mechanisms of cannabinoid action. We therefore conclude that methanandamide reduces infarct size in rat isolated

hearts by interaction with one or more novel sites of action that might involve a new cannabinoid receptor subtype.

The finding that methanandamide is cardioprotective in this model demonstrates its potential to be used as an adjunct of thrombolysis. Unlike in the global model of ischaemia (Chapter 4) where agonists were administered before ischaemia, the drug (methanandamide) was added at a clinically relevant time point (just prior to reperfusion) at which thrombolytics would be introduced to break down the occluding clot and therefore reperfuse the ischaemic area of the heart. However, further investigation needs to be carried out into the *in vivo* effects of methanandamide in this situation as cannabinoids are known vasodilators and therefore could potentially have adverse effects such as excessive hypotension. However, it is unlikely that a patient would be active (i.e. walking) when the drug was administered and therefore a drop in blood pressure would not present a big problem.

Chapter 6

General Discussion

6.0 Discussion

Cannabinoids have previously been shown to have a number of effects on heart rate including tachycardia as a result of Δ^9-tetrahydrocannabinoid and cannabinol treatment and bradycardia as the result of cannabidiol treatment in isolated hearts (Smiley *et al.*, 1976). However there have not been any investigations into the direct effects of cannabinoids on the isolated right atria. The study reported in this thesis has shown that cannabinoids have a direct effect on the sino-atrial node causing an increase in heart rate which was shown to involve an uptake and conversion pathway into products of the cyclooxygenase family such as prostaglandins or prostacyclins. In addition, when baseline heart rate was increased by a sympathomemetic, cannabinoid agonism caused negative chronotropy. The mechanism of this response remains unknown; however, the evidence suggests the involvement of the CB_2 cannabinoid receptor although the evidence is inconclusive because the CB_2 receptor selective antagonist, SR144528 was ineffective in blocking the negative chronotropic response.

Cannabinoids have potential therapeutic benefits over other well known cardioprotective agents such as adenosine because they have a minimal effect on heart rate. Adenosine, as with cannabinoids, can be produced as a retaliatory metabolite and also acts through G_i linked receptors (Mubagwa & Flameng, 2001). Adenosine has been shown to have a depressant effect on the sino-atrial node, with intravenous bolus administration causing sinus arrest (Belardinelli *et al.*, 1989).

Work as part of this thesis has also added further credence to that of previous studies implicating cannabinoids in cardioprotection (reduction in infarct size) in both a global

and regional ischaemia model of cardiac damage. Two previous studies which implicated endocannabinoids in the protection against ischaemia-reperfusion injury reported the involvement of cannabinoids in the protection afforded by lipopolysaccharide treatment (Lagneux & Lamontagne, 2001) and in the limitation of infarction induced by heat stress preconditioning (Joyeux *et al.*, 2002). In both studies SR144528 the CB_2 receptor antagonist inhibited the protective effects of the treatments indicating that activation of CB_2 receptors are involved. CB_2 receptor involvement in cardioprotection has also been reported in a more direct study by (Lepicier *et al.*, 2003) where palmitoylethanolamide and 2-arachidonoylglycerol but not anandamide were shown to improve recovery and reduce infarct size in a low-flow model of global ischaemia. A selective CB_1 agonist, ACEA and a selective CB_2 agonist, JWH-015 also limited infarction. However, the results of the Lepicier study are confusing because palmitoylethanolamide has no efficacy at either CB_1 or CB_2 receptors (Felder *et al.*, 1995) and was shown to be devoid of any activity when administered to isolated rat hearts (Ford *et al.*, 2002). Although this study did not identify the exact mechanism by which cannabinoids were mediating protection against damage caused by ischaemia-reperfusion it was able to eliminate a number of candidate receptors that do not appear to be involved including both the cloned cannabinoid receptors (CB_1 and CB_2) either individually or in combination or TRPV1. Uptake of the cannabinoids was also ruled out leading to the conclusion that cannabinoids were mediating protection through a novel site.

Novel sites of actions of cannabinoids have previously been reported. These include a non-CB_1, non-CB_2 endothelial receptor where anandamide and methanandamide but not

synthetic cannabinoids or Δ^9-tetrahydrocannabinoid have been shown to be agonists and which is sensitive to the CB_1 receptor antagonist SR141617A but at a higher concentration than for the CB_1 receptor (Chaytor *et al.*, 1999; Jarai *et al.*, 1999; Mukhopadhyay *et al.*, 2002; Wagner *et al.*, 2001a; Wagner *et al.*, 2001b; White *et al.*, 2001). The inhibitory action of SR141716A was only observed in the presence of an intact vascular endothelium and was lost on denudation. As a result it was proposed that there was an endothelial site distinct from CB_1 and CB_2 receptors but sensitive to SR141716A involved in the vasodilator effect of anandamide and methanandamide in the rat mesenteric circulation (Jarai *et al.*, 1999). It is possible that a similar mechanism may account for the actions of cannabinoids in the rat coronary circulation (Ford *et al.*, 2002). It is not believed that the endothelial receptor is responsible for the cardioprotection afforded in this study because the CB_2 receptor antagonist SR144528 blocked the protective effect and it has previously been demonstrated that the endothelial receptor is insensitive to this antagonist (Mo *et al.*, 2004).

A non-CB_1, non-CB_2 receptor on immune cells has also been proposed based on the finding that the analgesic effect of palmitoylethanolamide is blocked by the CB_2 antagonist SR144528, but not the CB_1 antagonist SR141716A (Calignano *et al.*, 1998; Jaggar *et al.*, 1998) even though palmitoylethanolamide does not bind to CB_2 receptors (Griffin *et al.*, 2000). This response was also not the result of TRPV1 activation because palmitoylethanolamide did not inhibit the capsaicin-induced pain (Calignano *et al.*, 2001).

Splice variants of CB_1 known as CB_1A and CB_1B have also been identified (Ryberg *et al.*, 2005; Shire *et al.*, 1995). Polymerase chain reaction revealed that CB_1 was not present in the heart and neither was the CB_1B splice variant, however, CB_1A was found (Ryberg *et al.*, 2005). This verifies results in a previous study which first identified the CB_1A variant and showed that both CB_1 and CB_1A mRNA was present in the heart (Shire *et al.*, 1995). Due to the low abundance of cDNA for both of the CB_1 splice variants it could be suggested that they do not contribute to cannabinoid pharmacology in different tissues. It is not believed that the splice variants are responsible for the cardioprotection seen or the effects on heart rate in this study because none of the known cannabinoid ligands of the CB_1 receptor including anandamide have any significant agonist activity at either of the variants (Ryberg *et al.*, 2005).

In silico searching of public patent data bases has recently revealed a potentially new cannabinoid receptor (GPR55) that displays high affinity for endogenous, natural and synthetic cannabinoids (Drmota, 2004). However, it is unlikely that this orphan G-protein coupled receptor is responsible for mediating the responses observed in the heart as SR141716A acts as a GPR55 agonist (Brown *et al.*, 2005; Brown & Wise, 2001) whereas responses to anandamide were antagonised in our study.

In the future investigation is required to establish the signal transduction pathways involved in the cardioprotective mechanism of cannabinoids (Figure 6.1). CB_1 receptors have been shown to be coupled to both phosphoinositide 3'-kinase and protein kinase B (Gomez del Pulgar *et al.*, 2000). Activation of both of these proteins has been shown to mediate the inhibition of ischaemia reperfusion-induced cardiac apoptosis

caused by insulin (Jonassen *et al.*, 2001). Therefore the inhibition of apoptosis induced by anandamide (Maccarrone *et al.*, 2000b) could be the result of activation of one of these pathways.

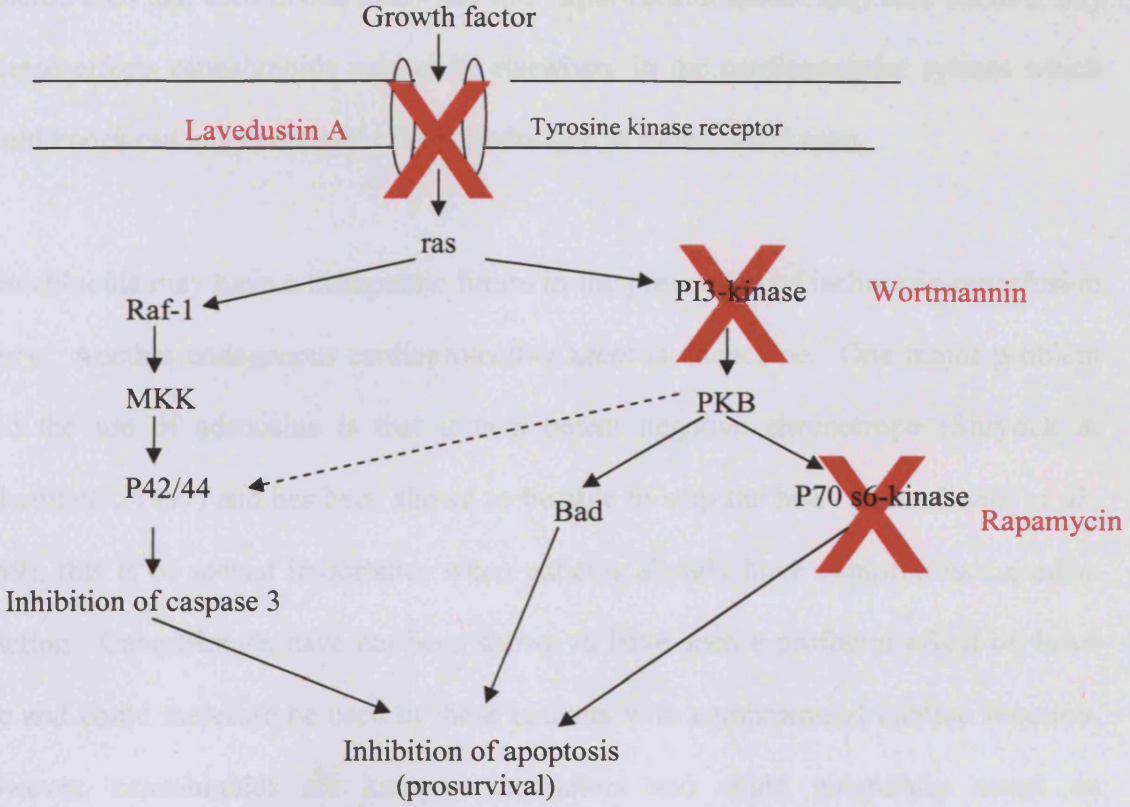


Figure 6.1 *Possible investigative pathway for the anti-apoptotic effect of cannabinoids. Lavedustin A (tyrosine kinase receptor inhibitor) and wortmannin (PI3-kinase inhibitor) would be used to see if cannabinoids act upstream of protein kinase B (PKB) and rapamycin (p70 s6-kinase inhibitor) would be used to investigate if cannabinoids affect prosurvival down stream of PKB where P70 s6-kinase promotes protein synthesis and the phosphorylation of Bad leads to the inhibition of apoptosis. Inhibitors are represented by X.*

In vivo work to ascertain the affect of cannabinoids on the whole body is also required. This is necessary because the relationship between cardiac function and blood pressure

is complex, and an *in vivo* model is the only way to accurately assess drug action on blood pressure, cardiac output and heart rate. An *in vivo* model would not only investigate the pharmacological effects of cannabinoids but would also be used to demonstrate that treatments to reduce ischaemia-reperfusion injury in a more complex situation than that used in this study and this experimental model may also uncover any adverse effects cannabinoids may elicit elsewhere in the cardiovascular system which would knock out any beneficial effects (reduction in infarct size) seen.

Cannabinoids may have a therapeutic future in the prevention of ischaemia-reperfusion injury. Another endogenous cardioprotective agent is adenosine. One major problem with the use of adenosine is that it is a potent negative chronotrope (Shryock & Belardinelli, 1997) and has been shown to be able to stop the heart (Belardinelli *et al.*, 1989), this is of special importance when patients already have compromised cardiac function. Cannabinoids have not been shown to have such a profound effect on heart rate and could therefore be used in those patients with compromised cardiac function. However, cannabinoids are known vasodilators and could potentially cause an unwanted decrease in blood pressure. Uptake inhibitors could then be used in conjunction with the cannabinoid to prevent this occurring. Although more experimental work is required I believe that cannabinoids have the potential as future therapeutic drugs for the treatment of ischaemia-reperfusion injury.

Not only could cannabinoids be a potential treatment for ischaemia-reperfusion injury but they could also be used for the clinical management of cardiogenic shock. Activation of vascular CB_1 receptors has been shown to contribute to severe

hypotension after experimental myocardial infarction and the selective CB_1 antagonist prevented post-myocardial infarction hypotension although this aggravated early endothelial dysfunction and worsened mortality. Therefore targeting cannabinoid biosynthesis, specific receptors and biological degradation systems may become a new strategy for the clinical management of cardiogenic shock (Wagner *et al.*, 2001a).

It is obvious that we are at the very beginning in our understanding of cannabinoid pharmacology and it is apparent through all the work in this thesis that there are responses in the heart that cannot be explained by activation of one or more of the currently characterised pathways. Until the full range of cannabinoid-related receptors is determined, problems related to attributing functions to cannabinoid molecules based on pharmacology will remain. It is equally clear that cannabinoid-based therapeutics may be of benefit in numerous clinical settings making the study of cannabinoids pharmacology a clear priority.

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