THE PHARMACOLOGICAL MODIFICATION OF REPERFUSION INJURY WITH PARTICULAR REFERENCE TO CALCIUM FLUXES IN THE ISOLATED RAT HEART.

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CONTENTS

ABBREVIATIONS USED IN THE TEXT

Units of measurement.

^{o}C	degrees Celsius
cm	centimeter
δ	delta
gm	gram
h	hour
kg	kilogram
L	liter
M	molarity (mmol/L)
m	meter
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mmol	millimole
ng	nanogram
sec	seconds
U	Unit
μg	microgram

Chemical compounds

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BAPTA	5,5'-difluoro 1,2-bis (2-aminophenoxy) ethane- N,N,N',N'-tetraacetic acid
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic adenosine 3'5'-monophosphate
CK	creatine kinase
Cl^{-}	chloride
CO_2	carbon dioxide
CPA	cyclopiazonic acid
CP	creatine phosphate
DMSO	dimethyl sulphoxide
EDTA	ethylenediamine-tetraacetic acid

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F-6-P	fructose-6-phosphate
G-6-P	glucose-6-phosphate
G-6-PDH	glucose-6-phosphate dehydrogenase
H^+	hydrogen
H_2O	water
HCL	hydrochloric acid
HCO_3	bicarbonate
Hg	mercury
HK	hexokinase
K^+	potassium
KOH	potassium hydroxide
LDH	lactate dehydrogenase
Mg^{2+}	magnesium
Na^+	sodium
NADPH	nicotinamide adenine dinucleotide phosphate -reduced form
NADP	nicotinamide adenine dinucleotide phosphate
O_2	oxygen
PCA	perchloric acid
PCr	phosphocreatine
PK	pyruvate kinase
TRIS	tris(hydroxymethyl) aminomethane hydrochloride
TTX	triton X100

Other abbreviations.

ANOVA	analysis of variance
AO	aortic output
CF	coronary flow
CO	cardiac output
ECG	electrocardiogram
HR	heart rate
LA	left atrium
LV	left ventricle
LVP	left ventricular pressure
LVDP	left ventricular developed pressure
NMR	nuclear magnetic resonance
SEM	standard error of the mean
SV	stroke volume

SWI	stroke work index
RPP	rate pressure product

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ABSTRACT

Myocardial reperfusion injury is thought to be caused by reperfusion induced i) cytosolic Ca^{2+} overload and/or, ii) the formation of oxygen derived free-radicals. At the start of this study, data implicating cytosolic Ca^{2+} overload in the genesis of reversible reperfusion injury were inconclusive. Although several workers have approached this problem by measurements of cytosolic calcium ions, it was my aim to examine the potential sources of such calcium overload.

The experiments reported in this thesis were therefore designed to examine the role of altered intracellular and transsarcolemmal Ca^{2+} fluxes in the genesis of reperfusion stunning and arrhythmias. The study was also aimed at elucidating the possible sources and entry pathways contributing to this proposed cytosolic Ca^{2+} overload.

In order to investigate the possible role of altered reperfusion Ca^{2+} fluxes in reperfusion injury, we exposed the isolated working, and Langendorff perfused rat heart model to ischaemia and reperfusion to induce reperfusion stunning and arrhythmias. Hearts were pre-treated (before ischaemia) or reperfused with pharmacological compounds, or by interventions known to enhance or inhibit intracellular or transsarcolemmal Ca^{2+} fluxes. The severity of reperfusion stunning (mechanical dysfunction) was measured by reperfusion aortic output, coronary flow and left ventricular pressure. The incidence of reperfusion ventricular arrhythmias was measured by the incidence of ventricular tachycardia and/or fibrillation. In selected studies, the metabolic status of hearts was evaluated using biochemical assays performed on myocardial tissue samples.

Data obtained in these studies indicate that increased Ca^{2+} fluxes through sarcolemmal L-type Ca^{2+} channels during early reperfusion exacerbate stunning, while inhibition of these fluxes with the Ca^{2+} antagonist drug nisoldipine or by Mg^{2+} or Mn^{2+} improve reperfusion function. These data also suggest that although interventions increasing Ca^{2+} fluxes early in reperfusion exacerbate reperfusion stunning, these same interventions improve reperfusion function when performed later.

The data also indicate that Ca^{2+} may enter the myocyte indirectly via activation of the Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchanger during reperfusion. Inhibition of Na^{+}/H^{+} exchange activity by HOE 694 during reperfusion attenuated reperfusion stunning and arrhythmias. Both activation of the Na^{+}/H^{+} (and Na^{+}/Ca^{2+}) exchanger and Ca^{2+} influx via the Ca^{2+} channel could contribute to reperfusion induced Ca^{2+} overload and subsequent injury.

The study also showed that altered intracellular Ca^{2+} oscillations play a role in reperfusion stunning and arrhythmias as shown by the use of the SR Ca^{2+} release channel blocker, ryanodine. Inhibition of the sarcoplasmic reticulum Ca^{2+} ATP-ase pump by two novel inhibitors, thapsigargin and cyclopiazonic acid, during ischaemia and early reperfusion improved reperfusion function and reduced the incidence of ventricular arrhythmias.

Finally, it seems unlikely that endothelin contributes to reperfusion stunning in this model. Despite the Ca^{2+} mobilising properties of this endogenous peptide, the levels of endothelin released by the isolated rat heart were to low to significantly exacerbate reperfusion stunning in this model. Exogenously administered endothelin was only detrimental to reperfusion myocardial

function when unphysiologically high concentrations of the peptide were infused into the heart during reperfusion.

Taken together, these data suggest that: 1) Ca^{2+} fluxes during early reperfusion (intracellular and transsarcolemmal) play a role in reperfusion injury, 2) that both the Ca^{2+} channel and Na^{+}/H^{+} exchange activity contribute to reperfusion injury by possibly contributing to cytosolic Ca^{2+} overload and that, 3) altered intracellular Ca^{2+} oscillations through the SR play a role in both stunning and arrhythmias.

Thus the proposal is that modulation of Ca^{2+} fluxes through either the sarcolemma or the sarcoplasmic reticulum, lessen reperfusion injury (stunning and arrhythmias). Although these data do not provide direct evidence of reperfusion Ca^{2+} overload, they support the concept that calcium ions play a role in the genesis of reversible reperfusion injury.

CHAPTER 1

INTRODUCTION

1.0 Historical Survey of Coronary Artery Occlusion and Reperfusion.

The first studies investigating the effects of coronary artery occlusion and reperfusion on myocardial function were performed in the late 1860s by an A Bezold who clamped the left coronary artery of the rabbit heart. He documented an occlusion induced decrease in heart rate with an eventual terminal "fibrillar activity" (probably ventricular fibrillation) of the left ventricle in 9 of the 20 hearts studied. He also noticed that in 7 of these hearts, normal "regular pulsations" (presumably normal heart rhythm) returned on removal of the coronary artery clamp (cited from Porter, 1894).

In 1894, Porter published data from studies using the canine model in which he ligated one or more coronary arteries to investigate the effect of these ligations on mechanical function (intraventricular pressure), time to mechanical failure, and heart rate. He noticed that the degree of mechanical failure of the heart was related to the number of arteries ligated (severity of ischaemia) and that coronary artery occlusion invariably altered heart rate and led to abnormal heart rhythm.

Later, Baumgarten (1899) who collaborated closely with Porter, wrote a manuscript dealing with myocardial infarction in which he concluded that ischaemia induced loss of contractile function in the heart could be restored by reperfusion of the experimentally occluded coronary artery. This is evidently one of the earliest documentations recognising the possible beneficial effects of reperfusion of the ischaemic heart.

These studies by Porter and Baumgarten were of great significance in the recognition of the clinical syndrome of ischaemic heart disease. In 1899, William Welch, a leading American pathologist declared, *"Thrombosis of the coronary artery is an affection of great clinical importance"* and, *"cardiac infarction is more common than would appear from the meagre attention usually given to the subject in text books"*. (cited from Fye, 1991). There was also a growing awareness that coronary occlusion was not invariably fatal. In 1901, Ludolf Krehl proposed that sudden coronary occlusion was not always fatal and that a severe attack of angina or a temporary attack of cardiac failure might be the result of coronary occlusion.

In 1912 James Herrick attributed myocardial infarction to a coronary thrombosis and delineated the clinical features of acute coronary thrombosis. These observations of the clinical features of coronary thrombosis attracted little attention while physicians sought an objective means of demonstrating that a patient's chest discomfort and associated symptoms were unequivocally cardiac of origin. The advent of electrocardiography in 1909, provided physicians with the confirmation they wanted and enabled them to confirm subjective clinical symptoms with an objective electrocardiogram.

The proposal by Herrick, that coronary thrombi caused myocardial infarction however sparked decades of debate concerning the causality of coronary thrombosis in myocardial infarction. However, in 1980, angiographic findings of DeWood and co-workers showed that obstructive coronary thrombi were present in the large majority (87%) of patients presenting with myocardial infarction. This finding finally gave conclusive evidence for coronary thrombi as the major cause of myocardial infarction.

In the late 1920s, the importance of cardiac arrhythmias after acute myocardial infarction was being recognised. In 1929, Samuel Levine published a book devoted to coronary thrombosis in which he addressed concepts such as "risk factors", and more importantly, cardiac arrhythmias associated with myocardial infarction. He noted that, "*The frequency with which they will be observed will depend entirely upon the care and frequency with which the patient is studied, for many of these disturbances are very transient, lasting only hours or might not even be detected except with more complicated methods of graphic representation of the heart beat, like the electrocardiograph*".

In the mean time, laboratory investigations were being conducted to elucidate the effects of coronary artery occlusion and reperfusion on the myocardium. In 1935 Tennant and Wiggins studied the effects of coronary artery ligation on mechanical function of the dog heart and noticed that reperfusion was accompanied by ventricular fibrillation in one of two dogs. The earliest study looking at the consequences of reperfusion on the myocardium was that of Blumgart, Gilligan and Schlesinger (1941) who studied the effects of coronary artery occlusion of varying times, followed by reperfusion, on infarct size. Although their objective was to determine the effects of ischaemia and reperfusion on infarct size in the dog model, they also documented ischaemic and reperfusion arrhythmias (ventricular fibrillation) during their study.

In 1960, Jennings and co-workers performed a similar study to determine the ischaemic time needed to induce irreversible injury in the canine model. They noticed that 25-30 minutes of ischaemia was sufficient to induce some tissue necrosis and that reperfusion after 20-21 minutes of ischaemia, resulted in reperfusion ventricular fibrillation (VF). Reperfusion VF was also the major cause of death in animals that did not survive the operative procedure. During

these investigations, Jennings and co-workers (1960) came to the conclusion that reperfusion may accelerate cell necrosis in the ischaemic heart. This is probably the earliest study that documented the possible deleterious consequences of reperfusion.

The concept of reperfusion injury was only fully developed by Rozenkranz and Buckberg (1983) when, in a series of studies, they showed that modified arresting and reperfusion solutions could improve the functional recovery of hearts arrested by cardioplegic solutions. In 1985, Braunwald and Kloner discussed the possible consequences of reperfusion on the ischaemic myocardium. They concluded that myocardial reperfusion could be considered a double edged sword, with distinct deleterious effects on severely ischaemic hearts, but when carried out early in the course of ischaemia, its effects are usually beneficial.

The findings of DeWood and co-workers (1980), together with some early experimental observations that the myocardium can be salvaged by reperfusion (Ginks *et al.*, 1972; Reimer *et al.*, 1977), lead to efforts to dissolve, mechanically compress, or surgically remove the offending thrombi in patients with acute ischaemic syndromes.

This was the beginning of an era where much emphasis and effort has gone into developing revascularisation techniques such as thrombolysis and fibrinolysis. Subsequent studies demonstrating that administration of both intravenous plasminogen activator (Collen *et al.*, 1984), and intracoronary streptokinase (Laffel and Braunwald, 1984) can successfully lyse thrombi in patients, raised hopes that timely reperfusion of the myocardium by such means could save jeopardised cells otherwise destined to become necrotic.

There are at present numerous international studies such as the ISIS-3 and -4 (International Study of Infarct Survival) and GISSI-2 trials involving thousands of patients being undertaken to develop and identify the best and most successful fibrinolytic and antithrombotic regimen for revascularisation of the ischaemic myocardium under clinical conditions.

1.1 Myocardial Ischaemia and Reperfusion: A Review of Definitions.

By definition, ischaemia exists "*whenever the flow of arterial blood through the diseased vessels is reduced to a volume below that required by the myocardium for adequate function*". From the metabolic point of view, ischaemia exists "*whenever the arterial blood flow is insufficient to provide enough oxygen to prevent intracellular respiration from shifting from aerobic to the anaerobic form*" (Jennings, 1970; Jennings and Yellon, 1992).

Ischaemia, if prolonged and severe enough, causes changes in the cellular ultrastructure in the form of mitochondrial swelling, membrane disruption, shortening of the sarcomeres, and an overall loss of contractile function (Jennings and Ganote, 1976; Schaper *et al.*, 1979). These changes are thought to be brought about by complex mechanisms involving depletion of energy stores (Neely *et al.*, 1973; Jennings and Ganote, 1976; Jennings *et al.*, 1983), accumulation of metabolic side-products (Neely *et al.*, 1973) including lipid metabolites (Corr, Gross and Sobel, 1984), membrane disruption (Schaper *et al.*, 1979), intracellular acidosis (Cobbe and Poole-Wilson, 1980), intracellular Ca^{2+} accumulation (Clusin *et al.*, 1983; Nayler *et al.*, 1988; Opie, 1989a), and oxygen derived free-radical activity (Hess and Manson, 1984; Kako, 1987).

After the early studies by Tennant and Wiggers (1935) and Blumgart, Gilligan and Schlesinger (1941) and also the later work of Jennings and co-workers (1960)

the two basic concepts of i) irreversible, and ii) reversible ***ischaemic*** injury developed. The former condition being a consequence of prolonged severe ischaemia, caused cell death (myocardial infarction), while the latter was associated with brief ischaemia and a rapid total recovery of the myocardium with reperfusion (Jennings *et al.*, 1960).

Although the phenomenon of ***ischaemic*** injury had been recognised for years, the concept of ***reperfusion*** induced injury only developed in the late 1970s and 1980s (Hearse, 1977; Becker and Ambrosio, 1987; Opie, 1989a; Hearse, 1990) when it was realised that reperfusion may in itself induce injury of the ischaemic myocardium.

Reperfusion injury occurs as a direct consequence of the reperfusion of ischaemic tissue (Hearse 1977; Jennings, Reimer and Steenbergen, 1985) and is defined as "*those metabolic, functional, and structural consequences of restoring coronary arterial flow that can be avoided or reversed by modification of the conditions of reperfusion*" (Rozenkranz and Buckberg, 1983). Despite the risk that reperfusion may worsen ischaemic injury, it is an absolute prerequisite for the survival and recovery of ischaemic tissue (Hearse, 1977; Braunwald and Kloner, 1985; Becker and Ambrosio, 1987; Opie, 1989b).

In the mid 1970s the effects of ischaemia on myocardial mechanical function were restudied, and a new phenomenon was documented. Reperfusion after brief ischaemia of 15 minutes elicited a condition where mechanical dysfunction was prolonged but eventually totally reversible (Heyndrickx *et al.*, 1975). Such a delayed recovery of mechanical function was later termed myocardial stunning (Braunwald and Kloner, 1982).

Stunning is defined as *"a persistent post-ischaemic mechanical dysfunction despite the absence of irreversible damage"* (Bolli, 1990). The important point in the definition is that no matter how severe the dysfunction is, it must be completely reversible. Stunning is therefore sublethal injury which is distinctly different from myocardial infarction which is the consequence of lethal ischaemic injury caused by an occluded artery. Recently, Bolli (1992) drew attention to an important point not previously emphasised when defining stunning. He made the point that for stunning to be diagnosed in patients, coronary blood flow must be known to be normal, or near normal. Contractile dysfunction in patients may otherwise be incorrectly diagnosed as being due to stunning when it is actually silent ischaemia.

A second consequence of ischaemic reperfusion lies in the development of ventricular arrhythmias, which, in their mildest form present as ventricular premature beats and in their most severe form as ventricular fibrillation (Figure 1.1). Ventricular fibrillation causes a collapse of mechanical pumping of the heart with a potentially fatal decrease in cardiac output and an increase in ventricular wall tension and energy consumption in the face of a lack of oxygen (Manning and Hearse, 1984). Because reperfusion after cardio-pulmonary bypass often predisposes to the development of reperfusion ventricular fibrillation, its prevention is crucial for the survival of the reperfusion salvaged myocardium (Manning and Hearse, 1984).

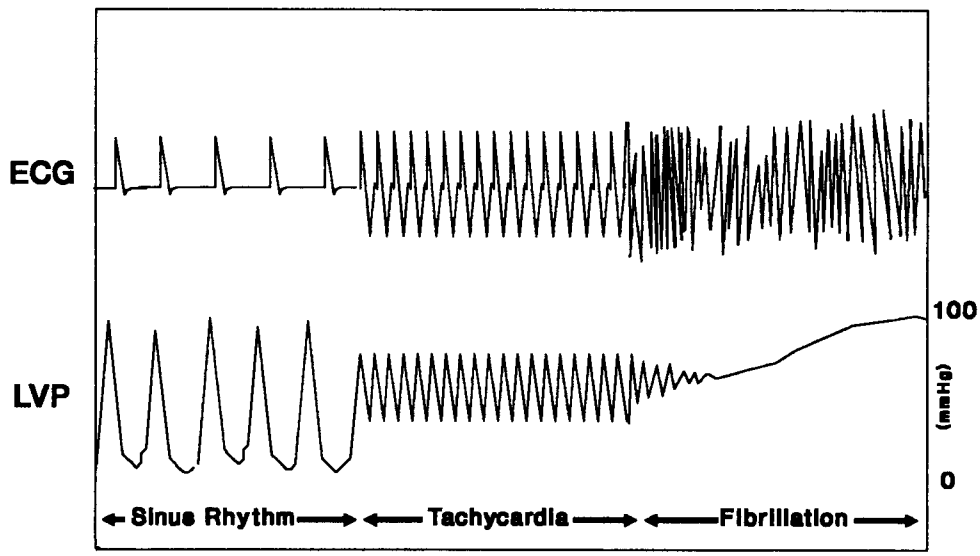

FIGURE 1.1 *A typical left ventricular pressure trace and electrocardiogram recorded during reperfusion of the isolated rat heart model. Note the loss of a left ventricular developed pressure with the transition from ventricular tachycardia to ventricular fibrillation (reproduced from traces obtained during experiments described in chapter 8).*

CHAPTER 2

CURRENT CONCEPTS IN REPERFUSION INJURY.

Controversy Concerning the Existence of Reperfusion Injury.

Since the early observations of Hearse (1977), the relevance of reperfusion injury to the clinical situation has been the topic of much debate and controversy. Decisive evidence for the existence of reperfusion injury would require either the appearance of a new phenomenon with reperfusion, or proof that reperfusion induced events can be prevented by interventions given at the onset of reperfusion. Because evidence for the existence of reperfusion injury was initially scant, it was suggested that reperfusion injury (in its various forms) may only occur under experimental conditions and thus be a laboratory artifact (Nayler and Elz, 1986), or, alternatively, that it may merely be an acceleration of damage which actually occurred during ischaemia (Hearse, 1991a). Most recently, Hearse, (1991b) proposed that stunning may merely be a form of convalescence during which time the heart recovers from the trauma induced by ischaemia. He suggests that the decreased mechanical function observed is merely the expression of a post-ischaemic "hangover", a phase of organ malfunction and recuperation that inevitably follows a period of trauma.

The investigations into the phenomenon of reperfusion stunning in man has been plagued by problems such as the limited accuracy of the methods available to non-invasively measure regional left ventricular function, the inability to quantify regional myocardial blood flow during ischaemia and reperfusion, and the uncontrolled influence of variables such as preload, afterload and adrenergic tone which have an effect on post-ischaemic dysfunction. The evolution of better non-invasive measuring techniques have helped the cardiovascular researcher determine that stunning does indeed occur in man after certain pathological conditions and clinical procedures.

Doubts concerning the existence and/or relevance of reperfusion injury in patients, have been negated by studies showing that reperfusion injury does indeed occur under clinical conditions (Patel *et al.*, 1988; Ferrari *et al.*, 1990; 1991; Renkin *et al.*, 1990; Breisblatt *et al.*, 1990; Braunwald, 1990; Kloner *et al.*, 1990). Bolli (1992), in a detailed review presented good evidence for the occurrence of reperfusion stunning in man after unstable angina, acute myocardial infarction with early reperfusion, open heart surgery, and cardiac transplantation.

These two pathological complications of reperfusion stunning and arrhythmias, are features of reversible reperfusion induced injury. Although both conditions are considered to be reperfusion related, the severity of reperfusion stunning and arrhythmias may be the ***net*** result of two distinct pathological processes. One being ischaemia related, and the other reperfusion related (Hearse, 1991b). Ischaemia related events and conditions may therefore contribute to the severity of reperfusion injury with interventions performed to act during ischaemia, decreasing ischaemic, and ultimately also reperfusion injury. Although stunning and reperfusion arrhythmias must be considered the two most important manifestations of reperfusion injury, this phenomenon also includes microvascular damage and reperfusion induced tissue necrosis (Opie, 1989b; Hearse, 1991a).

There are currently two major hypotheses implicating excess cytosolic Ca^{2+} and oxygen derived free-radicals in the genesis of reperfusion injury and which have thus far received considerable attention from numerous cardiovascular research groups.

2.1.1 Ca^{2+} and Reperfusion Injury.

The earliest study linking Ca^{2+} to reperfusion injury was performed by Jennings and co-workers (1960), who documented an increase in total tissue Ca^{2+} levels and the appearance of contracture bands with reperfusion of the canine myocardium. In 1974, Fleckenstein and co-workers introduced the concept that intracellular Ca^{2+} overload may induce myocardial fibre necrosis. Since then, numerous studies have shown that both reoxygenation after hypoxia (Allshire *et al.*, 1987), and reperfusion after ischaemia (Steenbergen *et al.*, 1987; Allen, Lee and Smith, 1988; Marban *et al.*, 1989) is associated with an increase in intracellular Ca^{2+} levels. These increases in intracellular Ca^{2+} levels in the heart have also been related to an increase in the incidence of reperfusion arrhythmias (Clusin 1983; Koretsune and Marban, 1989; Kihara and Morgan, 1991) and/or decreased mechanical function (Kusuoka *et al.*, 1987; Kitikaze, Weisman and Marban, 1988; Kusouka *et al.*, 1990).

Recently, the concept of Ca^{2+} overload induced reperfusion injury evolved further. It has been proposed that while a prolonged accumulation of total cell Ca^{2+} may be indicative of irreversible cell damage (Shen and Jennings, 1972a), an early transient increase in intracellular Ca^{2+} levels may cause, and be indicative of reversible reperfusion injury (Opie 1991c). Two hypotheses proposed by Opie (1991c) form the basis upon which the work documented in this thesis are based. First, he proposed that reperfusion after a brief period of ischaemia induces a transient Ca^{2+} overload (in the already Ca^{2+} loaded ischaemic heart) which contributes to reperfusion stunning (post-ischaemic mechanical dysfunction) and arrhythmias, and secondly, that elevated intracellular Ca^{2+} levels during ischaemia and early reperfusion may promote abnormal intracellular Ca^{2+} transients (oscillations between the sarcoplasmic

reticulum and the cytosol) which could exacerbate stunning and/or initiate and perpetuate arrhythmias (Figure 2.1). Figure 2.2 is a schematic representation of the possible cellular consequences of a loss in Ca^{2+} homeostasis as proposed by Nayler and Elz (1986).

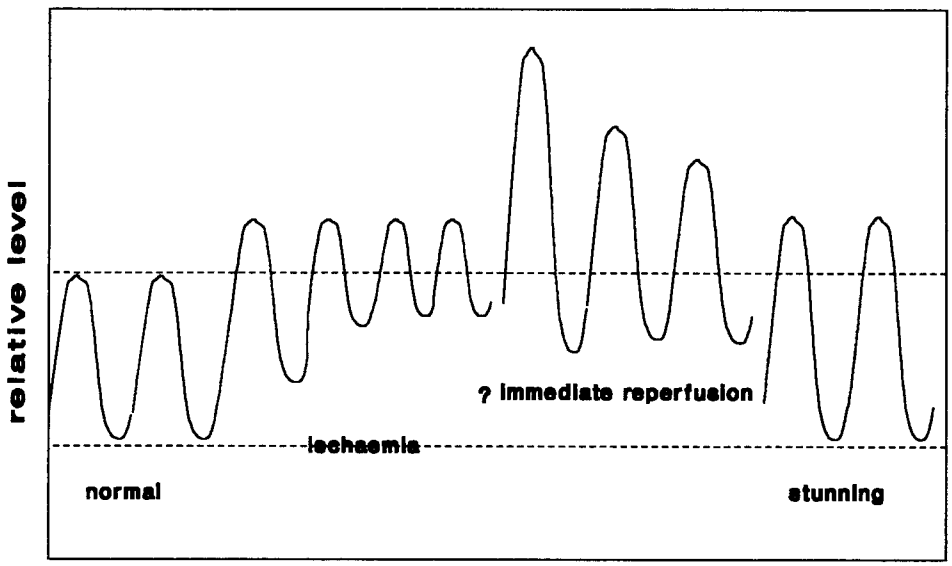

FIGURE 2.1 *Proposed changes in cytosolic Ca^{2+} levels during ischaemia and early and late reperfusion based on the data by Marban et al., (1990), Steenbergen et al., (1987), and Kusuoka et al., (1990) (figure reproduced from Opie, 1991c). Recent data published by Harada et al., (1994) confirm these proposals. This group has shown that cytosolic Ca^{2+} levels do indeed increase during ischaemia, with a subsequent further increase in cytosolic Ca^{2+} levels occurring during early reperfusion.*

Several studies provide evidence for the pathogenic role of Ca^{2+} in reperfusion stunning: 1) Perfusion of the isolated ferret heart with transient high concentrations of Ca^{2+} causes mechanical dysfunction (Kitakaze, Weisman and Marban, 1988), 2) Low Ca^{2+} reperfusion of the isolated ferret (Kusuoka *et al.*, 1987) and rat heart (Kuroda, Ishiguro and Mori, 1986) improves reperfusion

function and, 3) Ventricular fibrillation is accompanied by an elevated intracellular Ca^{2+} (Kihara and Morgan, 1991) and decreased post-fibrillatory mechanical function (Koretsune and Marban, 1989). Similarly, abnormal intracellular Ca^{2+} levels can be linked to arrhythmias as: 1) Strophanthidin induced ventricular fibrillation is associated with an increase in intracellular Ca^{2+} levels and oscillations (Kihara and Morgan, 1991), and 2) ventricular fibrillation is associated with an increase in intracellular Ca^{2+} levels measured in BAPTA loaded non-ischaemic ferret hearts (Koretsune and Marban, 1989).

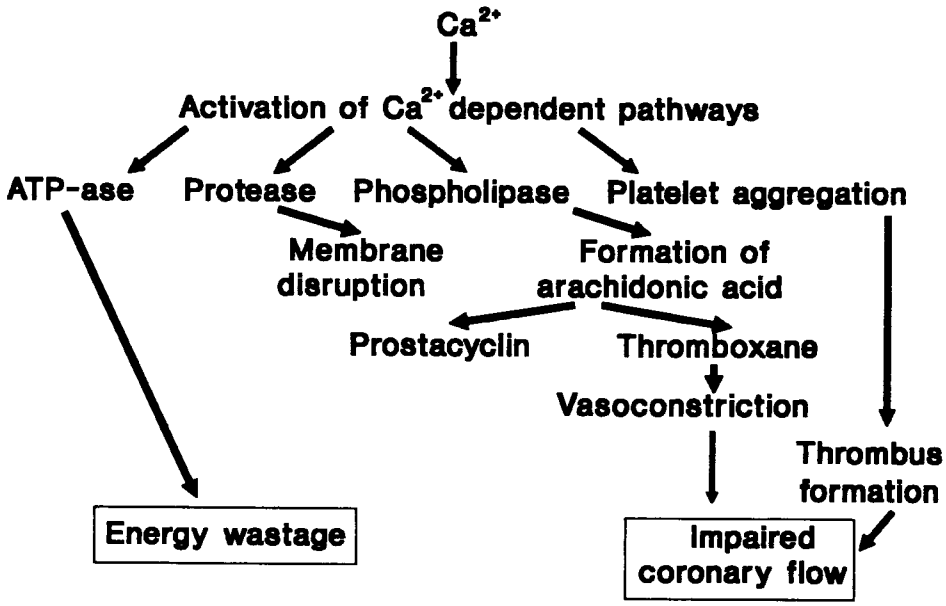

FIGURE 2.2 *Schematic representation of the direct consequences of loss of* Ca^{2+} *homeostasis during post-ischaemic reperfusion (figure reproduced from Nayler and Elz, 1986).*

2.1.2 Free-radicals and Reperfusion Injury.

Although Ca^{2+} has been implicated as a major factor in reperfusion injury, it is not the only cause of this type of injury (Bolli, 1990; Bolli *et al.*, 1990; Hearse 1991a, 1991b). A second and possibly equally important cause of reperfusion injury is the generation of oxygen derived free radicals during ischaemia and reperfusion (Hess and Manson, 1984; Myer *et al.*, 1985; Gross *et al.*, 1986). These oxygen derived free radicals are highly reactive molecules that may cause a variety of types of cell damage including phospholipid peroxidation and the degradation or cross-linking of protein or nucleic acid molecules (Halliwell and Gutteridge, 1985; Miki *et al.*, 1988; Gutteridge and Halliwell, 1990).

Free-radicals are normally produced by cells from about 5% of the available oxygen, but are broken down by naturally occurring free-radical scavenger enzymes in the normally functioning non-ischaemic myocyte (Hess and Manson, 1984). During ischaemia, these enzymes may be inhibited allowing increased accumulation of these intermediates.

During hypoxia and ischaemia, ATP metabolites such as xanthine, hypoxanthine and Ca^{2+} accumulate, leading to free radical formation (McCord, 1985). McCord (1985) proposed that intracellular Ca^{2+} accumulation may activate a protease, possibly calpain, which converts xanthine dehydrogenase to xanthine oxidase. In the presence of molecular oxygen, this enzyme will convert hypoxanthine to urate with the formation of free-radicals (Figure 2.3). This proposal has been confirmed by data showing that this process may be a significant contributor to oxygen free-radical formation in capillary endothelial cells in the isolated rat heart model (Chambers *et al.*, 1989). It must however be stressed that this mechanism appears to be species specific and may not relevant to man (Podzuweit *et al.*, 1988).

Reperfusion after an ischaemic episode is also associated with the generation of free-radicals such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH)(Mc Cord, 1985). These free-radicals could be formed by 1) increased activity of xanthine oxidase, which is active in dogs (Holzgrefe and Gibson, 1989; Przyklenk, Whittaker and Kloner, 1990) and the rat (Bernier, Hearse and Manning, 1986) but may not operate in man, 2) activation of neutrophils, 3) activation of the arachidonate cascade, 4) accumulation of reducing equivalents during oxygen deprivation, and probably most important, 5) derangement of the intramitochondrial electron transport system resulting in increased univalent reduction of oxygen (Hess and Manson, 1984).

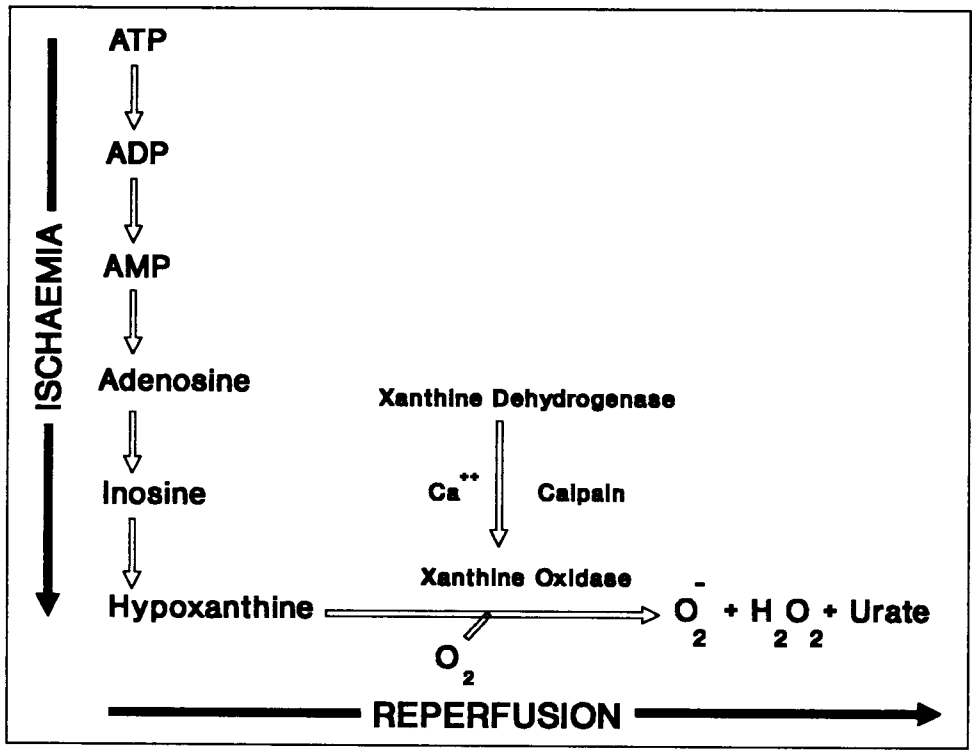

FIGURE 2.3 The proposed mechanism for the generation of superoxide and secondary species of active oxygen after ischaemia and reperfusion in the rat and dog heart (figure reproduced from McCord, 1985).

The respective quantitative contributions of free-radicals and Ca^{2+} to reperfusion injury has not been investigated, but it is suggested that they may be two facets of the same phenomenon (Bolli, 1990). It is possible that free-radicals cause membrane disruption, which facilitates abnormal Ca^{2+} redistribution in the cell (Figure 2.4) (Vandenplassche *et al.*, 1990). This Ca^{2+} redistribution may exacerbate cell damage and further promote free-radical formation (Hearse, 1991b).

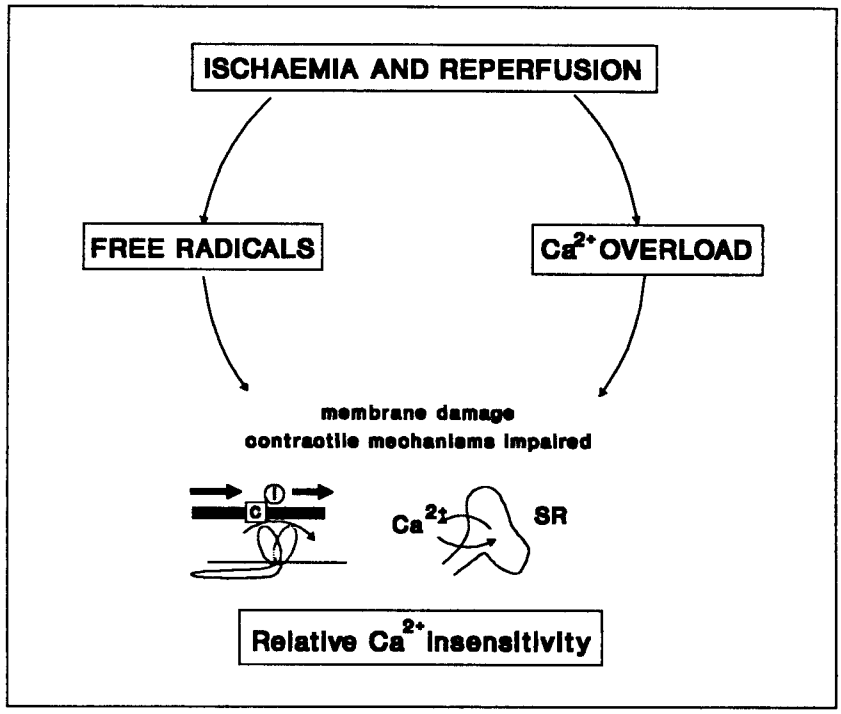

FIGURE 2.4 *Illustration of the two major mechanisms for reperfusion injury, which are believed to be by formation of oxygen derived free-radicals and* Ca^{2+} *overload. This scheme reconciles these apparently conflicting hypothesis, showing the proposed combined role of free radicals and* Ca^{2+} *overload in causing membrane damage and relative* Ca^{2+} *insensitivity of contractile mechanisms (actin-myosin interaction on left and sarcoplasmic reticulum on the right) (figure reproduced from Opie, 1989b).*

2.2 Factors Governing the Severity of Reperfusion Injury.

Although reperfusion injury is recognised as a reperfusion related pathology, conditions during ischaemia set the stage for reperfusion and thus contribute to the severity of reperfusion injury (Manning and Hearse, 1984; Opie, 1989b; Bolli, 1990; Opie, 1991c).

i) Ischaemic time. The incidence of reperfusion arrhythmias increases as the ischaemic time is increased up to 10-20 minutes after which there is a gradual decline in these arrhythmias (Manning and Hearse, 1984). This is consistent with the idea that ATP and viable (reversibly injured) myocardium are required for reperfusion arrhythmias to occur (Manning and Hearse, 1984; Coetzee and Opie, 1987; Opie, 1989b). Further prolongation of the ischaemic time, leads to depletion of ATP and cell necrosis, thus decreasing the likelihood of arrhythmias occurring.

Ischaemic time also markedly influences the severity of metabolic injury in the reperfusion period. As the duration of ischaemia is increased from 30-90 minutes, there is a marked increase in total tissue and mitochondrial Ca^{2+}, a worsening of ischaemic contracture and exaggerated enzyme release (Ferrari *et al.*, 1986b; 1988). Ischaemic injury measured as the percentage tissue necrosis, increases as the ischaemic time increases while tissue ATP and phosphocreatine (PCr) contents fail to recover during reperfusion after extended ischaemia (Schaper *et al.*, 1987; Schaper and Schaper, 1988). Also, the longer the ischaemic time, the more severe the ensuing mechanical abnormalities (Heyndrickx *et al.*, 1975; Preuss *et al.*, 1987). There is thus evidence that the longer the period of ischaemia, the more severe the ischaemic and subsequent reperfusion injury.

Consequences of brief and prolonged ischaemic episodes on viability of reperfused myocardium: When the heart is exposed to a relatively short ischaemic episode of a few minutes, it ultimately recovers (Braunwald and Kloner, 1982), resumes active tension generation, regenerates its endogenous energy rich phosphate reserves (Jennings *et al.*, 1985), and retains a fairly normal ultrastucture (Schaper *et al.*, 1979) upon reperfusion. Equally important is that there is no excess total tissue Ca^{2+} accumulation under these conditions (Shen and Jennings, 1972).

On the other hand, hearts reperfused after prolonged ischaemic episodes (30 minutes and more) are unable to regenerate depleted energy rich phosphates (Jennings *et al.*, 1985). These hearts become oedematous (Jennings *et al.*, 1985; Jennings *et al.*, 1990; Schaper *et al.*, 1979), gain Na^{+} and Ca^{2+} (Shen and Jennings, 1972), lose K^{+} (Kantor *et al.*, 1990; Carney *et al.*, 1989) and are electrically unstable (Opie, Nathan and Lubbe 1979; Manning and Hearse, 1984). There are also ultrastructural changes such as mitochondrial swelling and the development of contracture bands which are concomitant with intracellular Ca^{2+} accumulation (Schaper *et al.*, 1979). These hearts are irreversibly or lethally injured.

Ultrastructural changes during ischaemia and reperfusion in the reversibly injured myocardium: The ultrastructural changes observed late in the reversible phase of ischaemia (15 minutes) are marked but reversible on reperfusion (Jennings *et al.*, 1985). Such hearts show signs of glycogen depletion, marked relaxation of myofibrils, and alterations in mitochondrial structure. Matrix granules normally present in the mitochondria of control hearts are absent in ischaemic hearts and there is a degree of mitochondrial swelling with

disorganisation of cristae but no evidence of sarcolemmal disruption (Jennings *et al.*, 1985).

On reperfusion after 15 minutes ischaemia, there are no ultrastructural changes evident in the first half minute of reperfusion. At 3 minutes, there is however evidence of ultrastructural changes with further mitochondrial swelling, less glycogen in the sarcoplasm, and myofibrils remain relaxed. After 20 minutes reperfusion, ultrastructure is almost normal with glycogen present, nuclear chromatin evenly distributed and the mitochondria mostly indistinguishable from controls (Jennings *et al.*, 1985).

ii) Severity of ischaemia. The effect of the severity of ischaemia on reperfusion injury has not been well documented but it has been shown that increased heart rate worsens the severity of ischaemic injury as measured by infarct size (Schaper *et al.*, 1987) and incidence of reperfusion arrhythmias (Lederman *et al.*, 1987) in the canine model. The Ca^{2+} antagonist, diltiazem used during ischaemia decreased heart rate and the incidence of reperfusion arrhythmias but was ineffective when heart rate was maintained by pacing (Tosaki, Szekeresh and Hearse, 1987). The higher the ischaemic heart rate, the worse the ischaemic injury (Schaper *et al.*, 1987). Similarly, the greater the degree of myocardial hypoperfusion, the greater the reperfusion induced formation of free-radicals and the worse the reperfusion stunning (Bolli *et al.*, 1988a).

iii) Rate of reperfusion. There is some evidence to show that uncontrolled sudden, rather than controlled reperfusion exacerbates reperfusion injury. Reperfusion arrhythmias (Yamazaki *et al.*, 1986; Peng *et al.*, 1989) and function (Yamazaki *et al.*, 1986) can be improved by controlled staged reperfusion in the dog model. The beneficial effects of controlled reperfusion on reperfusion

arrhythmias may however be species specific as staged reperfusion in the isolated rat heart model merely delayed, but did not prevent the onset of reperfusion arrhythmias (Ibuki, Hearse and Avkiran, 1992).

2.3 Possible Ca^{2+} Sources and Entry Pathways Contributing to Elevated Cytosolic Ca^{2+} levels on Reperfusion.

The exact mechanisms responsible for increased cytosolic Ca^{2+} levels upon reperfusion are at present poorly understood and have not been well defined. Although Ca^{2+} may enter the myocyte through holes in the sarcolemma in the irreversibly damaged myocardium, this would be unlikely in the reversibly injured myocardium where ultrastructural evidence for membrane disruption is absent (Schaper *et al.*, 1979; Jennings *et al.*, 1985; Schaper and Schaper, 1988).

There are numerous potential sources of Ca^{2+}, and pathways via which extracellular Ca^{2+} may enter the myocyte and induce reperfusion stunning and/or arrhythmias. It may be secondary to a decrease in the ability of the sarcoplasmic reticulum (Krause and Hess, 1985a; 1985b) or mitochondria (Opie, 1989b) to take up and buffer excess Ca^{2+}, or due to activation of the Na^{+}/Ca^{2+} exchanger secondary to an ischaemia induced increase in cytosolic Na^{+} levels (Lazdunski *et al.*, 1985; Tani and Neely, 1989; Murphy, Smith and Marsh, 1988). Ischaemia induced acidification of the intracellular space may cause reperfusion induced activation of the Na^{+}/H^{+} exchanger which would in turn be expected to increase Na^{+}/Ca^{2+} exchanger activity (Figure 2.5). Whatever the mechanisms, Ca^{2+} influx at the onset of reperfusion appears to exceed the handling capacity of systems responsible for Ca^{2+} extrusion from the cytosol resulting in transient Ca^{2+} overloading.

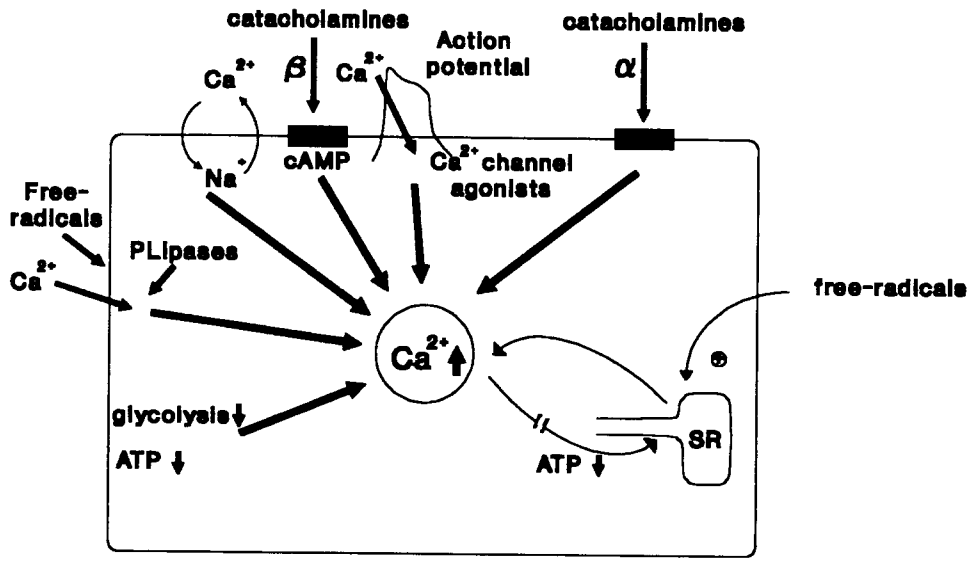

FIGURE 2.5 Illustration of proposed mechanisms of early gain of Ca^{2+} in the reperfusion period. Cytosolic Ca^{2+} may accumulate as a result of metabolic inhibition in the ischaemic period (reduced glycolysis and tissue ATP levels). During reperfusion, Ca^{2+} enters in a controlled manner through the Ca^{2+} channel or via Na^{+}/Ca^{2+} exchange. Uncontrolled Ca^{2+} entry may result from sarcolemmal damage, either from the activity of phospholipases or free radicals (figure reproduced from Opie, 1989b).

i) Ca^{2+} channels. The maximal rate of Ca^{2+} uptake on reperfusion after 30 minutes ischaemia is approximately 0.3 μmol/g/min for the first 30 minutes of reperfusion (Tani and Neely, 1989). Although this value is similar to that considered the normal influx rate in myocytes (0.1-0.3μmol/g/min (Lullmann, Peters and Preuner, 1983)), it does not exclude the possibility that the Ca^{2+} channel or the proposed "Ca^{2+} leak channel" (Cragoe, Post and Langer, 1993) may be partially responsible for the Ca^{2+} overload during reperfusion.

As Ca^{2+} flux through the Ca^{2+} channel is modulated by intracellular pH and tissue ATP availability, and 5-15 minutes of ischaemia decreases tissue ATP and pH levels sufficiently to inactivate these channels (Sperelakis, 1988), they would be expected to only contribute to Ca^{2+} influx during reperfusion when intracellular pH and ATP are normalised. Data showing that Ca^{2+} channel blockade (and transsarcolemmal flux inhibition) by verapamil or nitrendipine suppressed the inducibility of electrically induced ventricular fibrillation in the non-ischaemic isolated perfused rabbit heart (Merillat *et al.*, 1990) also indicate that abnormal Ca^{2+} fluxes through these slow channels may contribute to the genesis of ischaemic and reperfusion ventricular arrhythmias.

If however, the Ca^{2+} channels are responsible for the increase in Ca^{2+} on reperfusion, then Ca^{2+} channel antagonists given before, or at the onset of reperfusion should decrease reperfusion induced Ca^{2+} overload and reperfusion injury. Many investigators failed to show a decrease in Ca^{2+} overload and reperfusion stunning when Ca^{2+} antagonists were administered at the onset of ischaemia or reperfusion (Bourdillon *et al.*, 1985; Lamping and Gross, 1985; Ferrari *et al.*, 1986a; Przyklenk and Kloner, 1988). There are however studies that did demonstrate that Ca^{2+} antagonists given either before ischaemia (Watts, Koch and LaNoue, 1980; Nayler, Ferrari and Williams, 1980; Higgins and Blackburn, 1984; Poole-Wilson *et al.*, 1984; Watts, Maiorano and Maiorano, 1986; Warltier *et al.*, 1988; Nayler *et al.*, 1987; Nayler, Buckley and Leong, 1990; Watt *et al.*, 1990.), or during reperfusion (Higgins and Blackburn, 1984; Przyklenk and Kloner, 1988.) could attenuate Ca^{2+} accumulation and/or reperfusion injury. Ca^{2+} antagonists given *before* ischaemia may however also protect the ischaemic heart by reducing high energy phosphate consumption by 1) decreasing heart rate and/or, 2) decreasing contractility (Lange *et al.*, 1984) and may not necessarily act by decreasing reperfusion injury.

Indications are that the Ca^{2+} channel may contribute to reperfusion induced increases in cytosolic Ca^{2+} levels that have thus far remained undetected because of the relatively slow response time of Ca^{2+} measuring techniques.

ii) Ischaemic intracellular H^+ and Na^+ levels and the Na^+/H^+ exchanger. Ischaemic increases in intracellular Na^+ levels are thought to activate the Na^+/H^+ exchanger and cause increases in cytosolic Ca^{2+} levels during reperfusion. Unlike the Ca^{2+} channel, the Na^+ channel may be active during ischaemia as the Na^+ channel blocker, lidocaine given before ischaemia could attenuate ischaemia induced elevations in intracellular Na^+ concentration (Pike, Kitikaze and Marban, 1988). The fact that amiloride or dichlorobenzamil (a Na^+/Ca^{2+} exchange inhibitor) pretreatment did not decrease Na^+ accumulation during ischaemia, but did decrease Ca^{2+} accumulation on reperfusion may also suggest that the ischaemic increase in intracellular Na^+ levels is independent of the Na^+/H^+ and Na^+/Ca^{2+} exchangers (Tani and Neely, 1989; Tani, 1990).

Independent of the Na^+ channel, intracellular acidosis may also contribute to ischaemia induced elevations in the intracellular Na^+ concentration by activating the Na^+/H^+ exchanger (. Ischaemia causes a decrease in intracellular pH (Cobbe and Poole-Wilson, 1980) which may stimulate the Na^+/H^+ exchanger and account for the elevated intracellular Na^+ concentration during ischaemia (Lazdunski, Frelin and Vigne, 1985). This proposal that there may be a direct link between intracellular H^+ concentration and the elevation in Na^+ during ischaemia is controversial. Although an ischaemic elevation of the intracellular Na^+ concentration was proportional to the reduction in intracellular pH (Neubauer *et al.*, 1987), it has been suggested that the Na^+/H^+

exchanger may be inhibited at the low extracellular pHs occurring during ischaemia (Tani, 1990).

Despite the uncertainty surrounding the role of the Na^+/H^+ exchanger in Na^+ loading during ischaemia, there seems no doubt that the exchanger will be reactivated when extracellular pH is normalised during reperfusion. The intracellular/extracellular pH gradient created during early reperfusion will activate the Na^+/H^+ exchanger and indirectly contribute to elevated cytosolic Ca^{2+} levels on reperfusion (Figure 2.6). A role for this exchanger in reperfusion injury (presumably induced by cytosolic Ca^{2+} overload) was demonstrated by Kitikaze, Weisfeldt and Marban (1988) who reperfused ferret hearts with an acidotic reperfusion solution, thus inhibiting the Na^+/H^+ exchanger during early reperfusion, and improved subsequent reperfusion function. The concept that reperfusion induced Na^+/H^+ exchanger activation may contribute to the genesis of reperfusion ventricular arrhythmias was also supported by data showing that a stepped pH (6.6-7.4) restoration during reperfusion decrease the incidence of reperfusion ventricular fibrillation in isolated rat hearts (Avkiran and Ibuki, 1992).

Tani and Neely (1989) found that the increase in Na^+ levels during ischaemia was further elevated during reperfusion and remained high throughout the 30 minutes reperfusion. Although they (Tani and Neely, 1989) could eliminate this rise in reperfusion Na^+ influx by using pre-ischaemic glycogen depletion (thus decreasing ischaemia induced H^+ production) or amiloride treatment, this was only achieved when ischaemia was fairly severe. This reperfusion induced increase in cytosolic Na^+ was only evident when the ischaemia was so severe and prolonged that it caused a significant increase in tissue lactate, a decrease in pH and inactivation of Na^+,K^+-ATP-ase activity. This raises the question of

whether this increase in the Na^+ accumulation during reperfusion is relevant to the stunned heart where ischaemic times are relatively short (10-20 minutes) compared to the model of 30 minutes ischaemia used in the above-mentioned study.

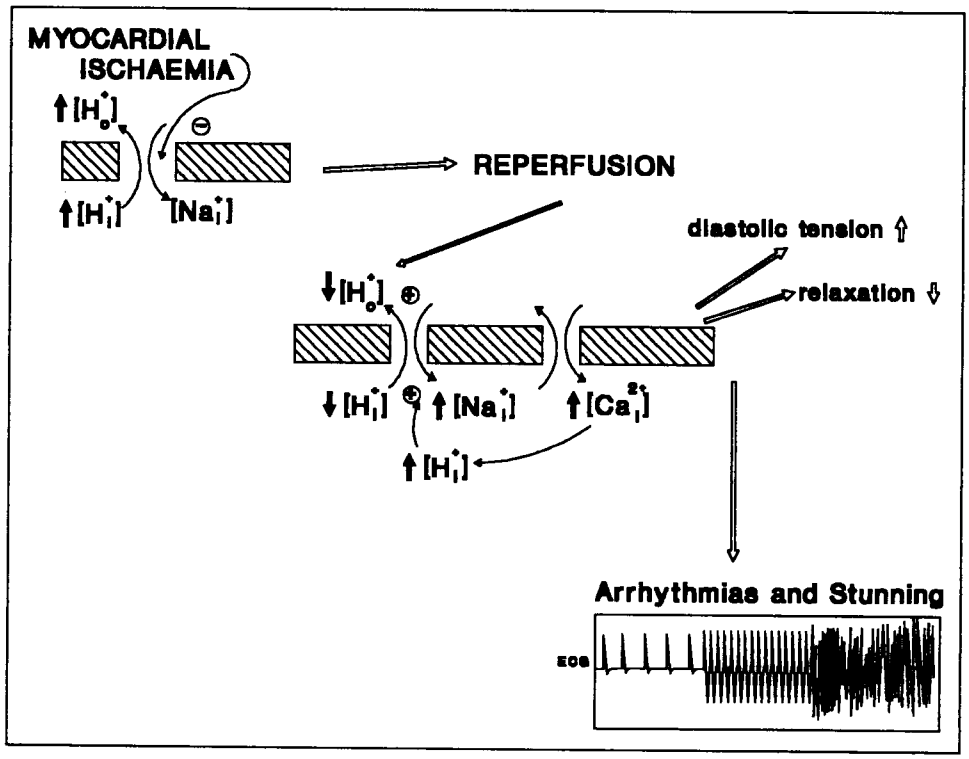

FIGURE 2.6 *Tentative scheme illustrating how recovery from ischaemia-induced acidosis may lead to myocardial stunning and arrhythmias. The hypothesis is that an accelerated* Na^+_o/H^+_i *exchange on reperfusion might be arrhythmogenic and/or contribute to reperfusion stunning. The reperfusion induced intracellular* Na^+ *loading may activate the* Na^+/Ca^{2+} *exchanger and induce a transient cytosolic* Ca^{2+} *overload (figure reproduced and modified from Dennis et al., 1990).*

One of the major problems facing researchers has been the unavailability of a specific pharmacologic inhibitor of the Na^+/H^+ exchanger. Although there are reasonable data to implicate the Na^+/H^+ exchanger in reperfusion injury, the nonspecificity of amiloride and its various derivatives implies that it may also have acted by various other mechanisms other than the Na^+/H^+ exchanger as

intended. Amiloride and its derivatives are all known to have effects on 1) the T-type Ca^{2+} channels (Tang, Presser, Morad, 1988), 2) the Na^{+} channels in various other tissue types (unknown for myocardium) (Benos, 1982) 3) Na^{+},K^{+}-ATP-ases and the Na^{+}/Ca^{2+} exchanger (Siegl *et al.*, 1984; Kennedy *et al.*, 1986). Data thus far published using these compounds is therefore imperfect and should be interpreted with caution.

iii) The Na^{+}/Ca^{2+} Exchanger. Although Na^{+}/Ca^{2+} exchange is inhibited by ischaemic acidosis (Philipson, Bersohn and Nishimoto, 1982) thus excluding a role for the exchanger during ischaemia, several researchers have proposed that the Na^{+}/Ca^{2+} exchanger may contribute to cytosolic Ca^{2+} overload during reperfusion (Grinwald, 1982; Renlund *et al.*, 1984) or hypoxia (Hagney *et al.*, 1992) and reoxygenation (Grinwald and Bosnahan, 1987; Murphy *et al.*, 1991; Marsh and Smith, 1991). The elevated cytosolic Na^{+} concentration observed during ischaemia (Pike, Kitikaze and Marban, 1988; Wilde and Kleber, 1986) led to the hypothesis that an ischaemia induced increase in intracellular Na^{+} concentration may activate the Na^{+}/Ca^{2+} exchanger on reperfusion and therefore indirectly contribute to Ca^{2+} overload.

Whilst it could be argued that the intracellular Na^{+} concentration would have to remain elevated for some time during reperfusion to significantly activate the Na^{+}/Ca^{2+} exchanger and increase cytosolic Ca^{2+} levels during reperfusion, the ischaemia induced Na^{+} "overloading" which is thought to be rectified by the Na^{+}/Ca^{2+} exchanger at the onset of reperfusion would contribute to a transient intracellular Ca^{2+} overload. It is also possible that the intracellular Na^{+} concentration may remain elevated for a couple of seconds after reperfusion as the Na^{+}/H^{+} exchanger, which is thought to be responsible for $\pm$35 % of the H^{+}

extrusion on reperfusion (Vandenberg *et al.*, 1993) extrudes H^+ in exchange for Na^+.

The most promising data to implicate the Na^+/Ca^{2+} exchanger in reperfusion Ca^{2+} overload and reperfusion injury were those showing that monensin (a Na^+ ionophore) increases intracellular Na^+ accumulation during ischaemia (Tani and Neely, 1989). Subsequent reperfusion after 30 minutes ischaemia was associated with an increase in $^{45}Ca^{2+}$ accumulation and a decrease in reperfusion mechanical function (increased stunning). Reperfusion of hearts with a low Ca^{2+}, high K^+ containing solution at the onset of reperfusion also improved reperfusion function, presumably by inhibiting Na^+/Ca^{2+} exchange activity (Tani and Neely, 1990a). These findings were also confirmed by data showing that reperfusion with a high Na^+ (Kusuoka, de Hurtado and Marban, 1993), or NiCl containing reperfusion solution (Lee and Allen, 1992), two interventions thought to decrease reperfusion Na^+/Ca^{2+} exchange activity improved reperfusion function.

iv) Sarcolemmal Ca^{2+} ATP-ase pumps, the sarcoplasmic reticulum and mitochondria. Although the contribution of the sarcoplasmic reticulum and mitochondria to reperfusion Ca^{2+} overload is thought to be relatively small, these organelles may act as Ca^{2+} buffers (Meno *et al.*, 1984; Opie, 1991c) and/or sources (Steenbergen *et al.*, 1990; Pesaturo and Gwathmey, 1990) during ischaemia and reperfusion. The normal function of these two organelles may therefore be important in the regulation of cytosolic Ca^{2+} levels during ischaemia and early reperfusion.

The role of mitochondria in Ca^{2+} homeostasis during brief ischaemia and reperfusion appears to be insignificant. After 45 minutes no-flow ischaemia,

mitochondria isolated from these hearts only have slightly elevated Ca^{2+} levels and generate ATP at only slightly reduced rates (Nayler, Ferrari and Williams, 1980). Continuation of ischaemia to 90 minutes causes a slight increase in mitochondrial Ca^{2+} content with reperfusion causing a massive influx of Ca^{2+} into the cells, and severe mitochondrial damage (Hearse, 1977). These findings were interpreted as being an indication that although Ca^{2+} is normally lower in the mitochondria than in the cytosol, ischaemia induced elevation of cytosolic Ca^{2+} levels may lead to mitochondrial Ca^{2+} loading with the mitochondria acting as a Ca^{2+} source on reperfusion (Pesaturo and Gwathmey, 1990). Pesaturo and Gwathmey (1990) proposed that the reoxygenation induced twitch prolongation and increase in Ca^{2+} transients seen in the ferret papillary muscle preparation was possibly the result of Ca^{2+} release from the mitochondria and/or sarcoplasmic reticulum on reoxygenation. They found that oligomycin and cyanide (thought to decrease Ca^{2+} release from mitochondria) and ryanodine (which acts on the SR Ca^{2+} release channel) could attenuate reoxygenation induced increased Ca^{2+} transients and twitch prolongation. Although these reoxygenation induced increases in cytosolic Ca^{2+} transients may not be directly relevant to reperfusion after ischaemia, these data may imply that these organelles have the capacity to act as Ca^{2+} sources on reoxygenation or possibly even reperfusion.

Very little is known about the possible contribution of the sarcoplasmic reticulum to Ca^{2+} induced stunning. In an early study, investigating the potential role of intracellular Ca^{2+} stores in ischaemic and reperfusion injury, Koomen, Schevers and Noordhoek (1983) preperfused hearts with a low Ca^{2+} containing perfusate and recorded improved mechanical function when reperfused. This was suggested to be due to pre-ischaemic intracellular Ca^{2+} store depletion which, may protect the heart by decreasing ischaemic intracellular Ca^{2+} overload.

Porterfield and co-workers (1987), found that ryanodine, in concentrations that induced sarcoplasmic reticulum Ca^{2+} release channel closure in the rat heart, could decrease morphologic changes and improve mechanical function on reperfusion. Also, pretreatment of isolated atrial tissue with caffeine or ryanodine decreased a simulated ischaemia induced increase in end-diastolic Ca^{2+} concentrations as measured with Indo-1 (Northover, 1991). Similarly, arresting the isolated rat heart with either high magnesium or potassium containing solution before an ischaemic period had different effects on the severity of ischaemic injury. Potassium arrest, thought to induce intracellular Ca^{2+} store (the SR) loading exacerbated ischaemic injury, while magnesium induced arrest, thought to deplete intracellular stores of Ca^{2+}, decreased the severity of ischaemic injury (Steenbergen *et al.*, 1990). Although no direct evidence for a role for the SR as a potential source of Ca^{2+} has been published, it does seem possible that ischaemia and/or reperfusion induced elevations in cytosolic Ca^{2+} levels may induce SR Ca^{2+} loading, and/or Ca^{2+} induced Ca^{2+} release from the sarcoplasmic reticulum. This may trigger: 1) futile Ca^{2+} oscillations, which would squander already deficient ATP levels, and/or 2) large Ca^{2+} oscillations (with large systolic peaks) which may further damage already jeopardised cellular components.

Because the sarcolemmal Ca^{2+} pumps are ATP dependent, it is possible that these pumps may fail to extrude excess Ca^{2+} during ischaemia and early reperfusion. The importance of sustained glycolysis during reperfusion was demonstrated by Jeremy and co-workers (1992) who showed that glycolytic inhibition at early reperfusion decreased reperfusion mechanical function, increased reperfusion end-diastolic pressure, and prolonged reperfusion cytosolic Ca^{2+} overload. It is however evident that under normal physiologic conditions, these pumps only work at half their maximal rate (Carafoli, 1985) and would be

expected to be capable of responding to the elevated cytosolic Ca^{2+} levels under pathophysiological conditions. Also, the sarcolemmal Na^{+}/Ca^{2+} exchanger has a much larger capacity to move Ca^{2+} than the ATP-ase pump and should therefore be capable of compensating for the reduction in function of these ATP-dependent pumps (Carafoli, 1985). The sarcolemmal ATP-ase pumps therefore appear to play an insignificant role in Ca^{2+} homeostasis during reperfusion.

One of the reasons the role of the SR in reperfusion stunning and arrhythmias has not been further investigated, is the lack of consensus among researchers on the mechanism of action of ryanodine and other SR specific compounds on the SR Ca^{2+} release channels. Some researchers believe ryanodine acts by opening the release channel (Rousseau, Smith and Meisner, 1987), while a second group believes it closes these channels (Marban and Weir, 1985; Inui *et al.*, 1988). These controversies have restricted numerous studies and rendered data obtained using these compounds inconclusive.

v) Indirect influence of endothelin on Ca^{2+} fluxes. Although endothelin is not a source of Ca^{2+}, the endogenous 21-amino-acid peptide has been reported to have multiple biological actions (Yanagisawa *et al.*, 1988). It contracts vascular and non-vascular smooth muscle and is known to exert a positive inotropic action on atrial and ventricular muscle (Nayler, 1990). Endothelin, by stimulating the PI (phosphoinositol) pathway, increases cytosolic IP_3 (inositol triphosphate) levels which stimulate SR IP_3 receptors to release Ca^{2+} into the cytosol (Nayler, 1990). By virtue of its Ca^{2+} mobilising properties (Vigne *et al.*, 1990; Nayler, 1990), endothelin may also contribute to cytosolic Ca^{2+} overloading and reperfusion injury (Opie *et al.*, 1989; Nayler, 1990)

It has been found that ischaemia caused a time dependent externalisation of endothelin-1 binding sites (Liu, Casley and Nayler, 1989), thus increasing the number of receptors available for the interaction with blood borne endothelin. A possible role for endothelin has also been suggested by data that shows that circulating endothelin levels increased within hours of onset of myocardial infarction in humans (Miyauchi *et al.*, 1989; Salminen *et al.*, 1989; Yasada *et al.*, 1990). There is thus good evidence to suggest that endothelin may play a role in the pathogenesis of ischaemic and reperfusion injury.

2.4 Proposed Mechanisms Underlying Decreased Mechanical Function in Stunning.

The exact mechanisms by which the proposed transient cytosolic Ca^{2+} overload causes contractile dysfunction is unknown, but may be related to cellular damage caused by Ca^{2+} activated phospholipase and/or other degradative enzyme (Opie, 1989a; Marban *et al.*, 1989; Hearse, 1991a) which may have an effect on contractile proteins. It is also believed that Ca^{2+} may activate the production of highly reactive oxygen free-radicals via xanthine oxidase in certain species (Mc Cord, 1985; Hearse, 1991a).

Since it became evident that stunning was not due to severe morphological damage and the stunned heart showed reversible ultrastructural changes, the mechanisms responsible for the characteristic decrease in mechanical function have been elusive. Of the numerous proposed hypotheses, many have been disproved while others remain unresolved.

i) Inadequate high energy phosphate production. An early proposal was that the stunned heart had incompetent mitochondria, unable to synthesise adequate ATP to support normal mechanical function which was possibly related to an

inadequate supply of adenine nucleotide precursors (Braunwald and Kloner, 1982; Reimer, Hill and Jennings, 1981). Early studies showed that the stunned heart had low ATP levels (Kloner *et al.*, 1983; Ellis *et al.*, 1983a; Reimer, Hill and Jennings, 1981) and that the ATP levels increased with a similar time course as recovery of function (Kloner *et al.*, 1983; Ellis *et al.*, 1983a). These findings were later disproved by reports showing that not only was there no correlation between tissue ATP levels and mechanical function (Taegtmeyer *et al.*, 1985; Kusuoka *et al.*, 1987), but there were also normal or supranormal phosphocreatine levels present in the stunned heart (Kloner *et al.*, 1983; Taegtmeyer, Roberts and Raine, 1985; Kusuoka *et al.*, 1987; Przyklenk and Kloner, 1986). Also, the PCr/P_i ratio (which is an indication of the balance between energy utilisation and production), was higher than baseline in the stunned heart (Kusuoka *et al.*, 1987). The latter finding would imply that the mitochondria of the stunned hearts were intact and functional. Another factor which indicated that ATP depletion was not the major cause of the decreased mechanical function was that stunned hearts were still able to respond to positive inotropic stimuli and perform mechanical work at levels above their pre-ischaemic baseline level (Arnold *et al.*, 1985; Becker *et al.*, 1986; Ambrosio *et al.*, 1987; Mercier *et al.*, 1982; Ellis *et al.*, 1983b; Ito *et al.*, 1987). These increases in mechanical function were not accompanied by a decrease in ATP or phosphocreatine levels and had no detrimental effect on subsequent mechanical function (Ambrosio *et al.*, 1987; Arnold *et al.*, 1987) In summary, all indications are that the decreased mechanical function associated with stunning is in no way related to total tissue high energy phosphate levels in the heart.

ii) Impaired energy utilisation by myofibrils. Another possible cause of decreased mechanical function was proposed by Greenfield and Swain (1987) who reported a decrease in creatine kinase activity after 15 minutes of ischaemia.

They noted a decrease in the free ADP used by the myofibrillar creatine kinase to produce ATP at the contraction site. This hypothesis is however questioned as it does not explain the contractile and metabolic reserve of the stunned heart. The fact that inotropic interventions used by some researchers could still elicit above normal mechanical work would suggest that the myofibrillar creatine kinase is adequate to run the myofibrillar ATP-ase reaction at normal levels even when the heart is stunned.

iii) SR dysfunction and excitation-contraction uncoupling. Although very little is known about the role of the sarcoplasmic reticulum in the decreased mechanical function associated with stunning, Krause and co-workers (1989) found that the ATP-ase pumps, of SR vesicles isolated from the stunned dog heart, exhibited a reduced capacity to transport Ca^{2+} into the vesicle. These findings were ascribed to a decreased Ca^{2+} ATP-ase pump capacity to sequester Ca^{2+} (Krause *et al.*, 1989; Limbruno *et al.*, 1989). The mechanism responsible for this decreased ATP-ase pump activity is not known but may be due to ischaemia induced changes in the characteristics of phospholamban (Schouten *et al.*, 1989). SR fractions from the stunned heart exhibited a decrease in ^{32}P incorporation in phospholamban which correlated well with the decrease in Ca^{2+} pump activity.

The hypothesis of dysfunction of the ATP-ase pump, although attractive, is difficult to reconcile with the finding that the stunned heart exhibited increased Ca^{2+} transients despite the decreased mechanical function (Kusuoka *et al.*, 1990). If we assume that the sarcoplasmic reticulum is responsible for intracellular Ca^{2+} transients associated with diastole and systole, then the sarcoplasmic reticulum Ca^{2+} ATP-ase pumps appear to be normal in the intact stunned heart. These changes in the SR Ca^{2+} ATP-ase pump activity may thus

be related to the preparation used and/or technique used to isolate the SR vesicles from the stunned myocardium.

Not only the Ca^{2+} uptake mechanisms, but also the Ca^{2+} release channels have been implicated in stunning. It has been suggested that the apparent decreased Ca^{2+} uptake capacity previously seen in post-ischaemic stunned SR vesicles was not due to a decreased Ca^{2+} uptake rate but an increased release through ryanodine sensitive channels (Feher, LeBolt and Manson, 1989). This would imply that the Ca^{2+} may leave the SR of the stunned heart faster than the Ca^{2+} ATP-ase pumps can sequester it thus leaving the SR Ca^{2+} depleted. Recently, two independent research groups reported that not only Ca^{2+} uptake, but also Ca^{2+} release from the sarcoplasmic reticulum was impaired in stunned heart ventricular homogenates (Hohl, Garleb and Altschuld, 1992; Davis, LeBolt and Feher, 1992). There is thus conflicting evidence concerning the proposal that excitation-contraction uncoupling occurs due to the inability of the sarcoplasmic reticulum to remove and/or supply adequate Ca^{2+} to the contractile proteins.

Despite data showing that there is no link between tissue ATP levels and reperfusion stunning (Taegtmeyer *et al.*, 1985; Kusuoka *et al.*, 1987), the possibility of ATP depletion in direct proximity of the SR cannot be ruled out. If ATP were compartmentalised in the myocyte (Bricknell, Daries and Opie, 1981), then inadequate ATP in direct proximity of the SR may be responsible for a decreased ATP-ase pump function. The SR Ca^{2+} ATP-ase pump is responsible for 50-80% of the cyclic Ca^{2+} sequestration from the cytosol (Bers, Bridge and Spitzer, 1989) and may exacerbate ATP depletion induced contracture (Barry *et al.*, 1987). The latter group proposed that an increase in cytosolic Ca^{2+} (as occurs during ischaemia) could induce SR Ca^{2+} loading which may hasten ATP depletion by activation of the ATP-ase pumps in the energy deprived myocyte

(Barry *et al.*, 1987). More recently, it was also shown that the dissociation between reperfusion mechanical function and oxidative metabolism (Laster *et al.*, 1989) could be attenuated by ruthenium red treatment of the reperfused isolated rat heart (Benzi and Lerch, 1992). Benzi and Lerch (1992) proposed that an increased energy expenditure by intracellular Ca^{2+} transport systems may be involved in the mechanisms underlying the dissociation between left ventricular performance and myocardial oxidative metabolic rate during reperfusion. The importance of the SR, and its ATP-ase pumps in reperfusion stunning may therefore be underestimated.

iv) Damage to extracellular collagen matrix. Although stunning occurs in hearts in the absence of myocyte necrosis, there is the concept that this decreased mechanical function may be related to damage to the extracellular collagen matrix which is responsible for mechanical coupling of myocytes. Collagen responsible for mechanical coupling between cells is disrupted and thus allows elasticity in the myocardial tissue (Zhao *et al.*, 1987; Carney *et al.*, 1989). It has recently become evident that although stunning can be linked to collagen breakdown, this breakdown is model dependent and occurred when the heart was subject to repeated bouts of ischaemia but not when a single ischaemic episode was used to induce stunning (Whittaker *et al.*, 1989). Collagen breakdown during ischaemia and early reperfusion may therefore only contribute to reperfusion stunning when multiple ischaemic episodes are used to induce stunning.

v) Decreased myofibrillar sensitivity to Ca^{2+}. As the availability of cytosolic activator Ca^{2+} does not seem to be at fault (Kusuoka *et al.*, 1990), it has been proposed that stunning may be due to decreased sensitivity of the contractile proteins to normal cytosolic Ca^{2+} levels (Kusuoka *et al.*, 1987; Kihara,

Grossman and Morgan, 1989; Allen, Lee and Smith, 1989; Marban *et al.*, 1990; Hofmann, Miller and Moss, 1993). Kusuoka and co-workers (1987) found that the myocardium of stunned isolated perfused ferret hearts were less responsive to extracellular Ca^{2+} added to the perfusate. These findings would imply that there is a myofilament Ca^{2+} insensitivity in the stunned heart in the presence of normal cytosolic Ca^{2+} levels. They also found that the maximal Ca^{2+} activated force (F_{max}) the stunned heart could generate was lower than in the normal heart. This decrease in the F_{max} of the stunned heart was later confirmed by Carrozza and co-workers (1992). They however report no decrease in the Ca^{2+} sensitivity of the myofilaments of the stunned heart. They proposed that the decreased F_{max} observed in the stunned heart may be due to an accumulation of metabolites such as inorganic phosphates rather than to decrease in myofilament sensitivity as had previously been suggested. Numerous studies have also shown that positive inotropes (Becker *et al.*, 1986; Mercier *et al.*, 1982; Ellis *et al.*, 1983) can still elicit normal and above normal myocardial contraction in the stunned heart which would argue against the concept that the stunned heart was insensitive to Ca^{2+}.

The major criticism of the studies on the Ca^{2+} response of the stunned heart was that they were done on *in vitro* models. The *in vivo* stunned heart however also retains a normal contractile reserve with an elevated intracoronary Ca^{2+} concentration eliciting normal contractile function in the stunned dog heart (Ito *et al.*, 1987). Whatever the mechanism of decreased mechanical function of the heart, interventions that increase cytosolic Ca^{2+} levels in the already stunned heart are able to improve mechanical function. The major problem therefore appears to be an impairment of Ca^{2+} function.

2.5 Cytosolic Ca^{2+} and Stunning.

The measurement of intracellular Ca^{2+} levels in the heart has been a major challenge to the cardiovascular scientist. Since the early 1970s, when it first became possible to measure changes in intracellular Ca^{2+} levels in actively contracting myocytes and the whole heart by bioluminescent, fluorescent and nuclear magnetic resonance measuring techniques (Snowdowne, Ertel and Borle, 1985; Steenbergen *et al.*, 1987; Koretsune and Marban, 1990; Marban *et al.*, 1990; Kihara, Grossman and Morgan, 1989; Lee *et al.*, 1988), these measurements have been controversial.

One of the first studies that successfully measured Ca^{2+} levels during ischaemia in the intact heart was performed by Steenbergen and co-workers (1987). Using nuclear magnetic resonance (NMR) techniques and the Ca^{2+} probe BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-teatraacetic acid) in an isolated rat heart model, they showed that the cytosolic Ca^{2+} concentration was elevated within 10 minutes of the onset of ischaemia. These data were later extended by Lee and co-workers (1987) who showed that the rise in cytosolic Ca^{2+} was very rapid and occurred within 30 seconds of the onset of ischaemia in the isolated Indo-1 loaded rabbit heart. A major weakness of these Ca^{2+} measuring techniques are that especially quin-2 and F-BAPTA, buffer Ca^{2+} and severely depress contractile function of the myocyte. To compensate for the Ca^{2+} buffering capacity of these Ca^{2+} indicators, some researchers have been compelled to use abnormally high perfusate Ca^{2+} concentration of up to 8 mM (Marban *et al.*, 1990). These high Ca^{2+} concentrations in the perfusate may have significant effects on Ca^{2+} fluxes and data obtained under these conditions should be interpreted with caution. Also, BAPTA measurements have to be performed at low temperatures ($30^{o}C$) which decreases myocardial metabolic rate and the severity of ischaemia.

With the evolution of intracellular Ca^{2+} measuring techniques, Kihara, Grossman and Morgan (1989) measured changes in the cytosolic Ca^{2+} concentration with aequorin in intact isolated ferret hearts under conditions of hypoxia and true global ischaemia. Under hypoxic conditions, both diastolic ventricular pressure and cytosolic Ca^{2+} levels were elevated. During ischaemia however, Ca^{2+} levels increased substantially but ventricular pressure declined (Kihara, Grossman and Morgan, 1989). Compounds such as aequorin, a bioluminescent dye which emits light when bound to Ca^{2+} is not only sensitive to Ca^{2+} but also to Mg^{2+}, an ion the concentration of which is also elevated in the ischaemic myocardium. Also, aequorin has to be injected into the cell by methods that may alter sarcolemmal membrane integrity and permeability (Kihara, Grossman and Morgan, 1989).

An early study by Lee and co-workers (1988) using the fluorescent Ca^{2+} indicator indo-1 in the intact heart showed increases in diastolic and systolic Ca^{2+} transients in the ischaemic heart. These increased light signals were assumed to originate from the ischaemic myocytes. This data was later challenged by Lorell and co-workers (1990) who showed that the endothelial cells of the myocardium also contain elevated intracellular Ca^{2+} levels under certain conditions and could contribute to the Ca^{2+} signal seen during normal and ischaemic conditions in studies using the intact heart model. Indo-1 also fluoresces when bound to NADH which may complicate interpretation of the indo-1 fluorescence signal measured under control and ischaemic conditions when the level of these metabolites may differ. Compounds such as Indo-1 are loaded into the cell in the ester form (Indo-1-AM). Incomplete intracellular de-esterification of the AM form of Indo complicates calibration with no satisfactory calibration technique presently available (Lee *et al.*, 1988).

A further defect of these studies is that the intracellular Ca^{2+} levels measured in the intact heart are only a reflection of the levels of Ca^{2+} in the epicardium and not of the presumably more ischaemic endocardium. Figueredo and co-workers (1993) reported a difference in the endo- and epicardial diastolic intracellular Ca^{2+} levels during low flow ischaemia in the rat heart. The intracellular Ca^{2+} concentration data collected from the whole heart preparation are thus imperfect and may not be representative of intracellular Ca^{2+} levels throughout the whole myocardium.

More recent studies using BAPTA and NMR techniques to measure intracellular Ca^{2+} levels in the ischaemic rat (Steenbergen *et al.*, 1990) and ferret heart (Marban *et al.*, 1989) confirm that cytosolic Ca^{2+} levels, and transients rise during ischaemia and that intracellular Ca^{2+} unavailability is not the cause of the decreased mechanical function seen in the ischaemic heart. This apparent myofilament insensitivity to Ca^{2+} may be due to competitive binding between H^{+} and Ca^{2+} during ischaemia induced intracellular acidosis (Chapman, 1983, 1984; Barry *et al.*, 1987).

Although there is conclusive evidence that cytosolic Ca^{2+} levels rise during ischaemia, the mechanism for this increased Ca^{2+} influx remains obscure. Studies using a model of simulated ischaemia in the isolated ferret ventricular papillary muscle preparation reported a striking rise in cytosolic Ca^{2+} levels, diastolic Ca^{2+} oscillations and Ca^{2+} transients (Allen, Lee and Smith, 1988, 1989). Because similar results were observed when hearts were perfused with high concentrations of lactic acid, they concluded that the rise in cytosolic Ca^{2+} was caused by ischaemic acidosis in their model of simulated ischaemia (Allen, Lee and Smith, 1989).

Cytosolic Ca^{2+} transients and stunning. When Fleckenstein and co-workers (1974) proposed that cytosolic Ca^{2+} overload may be due to high energy phosphate deficiency, they speculated that Ca^{2+} dependent ATP-ase pump activation during ischaemia may contribute to ATP depletion. Later studies showing large increases in Ca^{2+} transients during ischaemia (Lee *et al.*, 1987, 1988; Lee and Allen, 1992) and reperfusion (Kusuoka *et al.*, 1990) led some researchers to propose that these abnormal transients may exacerbate reperfusion stunning by causing ATP depletion (wastage) (Barry *et al.*, 1987; Kusuoka *et al.*, 1990) or by damaging the already jeopardised contractile apparatus during ischaemia. In a study of simulated ischaemia, the maximal amplitude of Ca^{2+} transients during ischaemia, was approximately 339 % of the control value after 18 minutes ischaemia (Lee and Allen, 1992). These large oscillations are thought to be caused by elevated cytosolic Ca^{2+} levels, causing SR Ca^{2+} loading and spontaneous SR Ca^{2+} release induced by intracellular acidosis (Orchard *et al.*, 1987). The increased Ca^{2+} transients (diastolic and systolic) seen in the reperfused, already stunned heart may also cause ATP wastage and contribute to the decreased mechanical function of the stunned heart (Kusuoka *et al.*, 1990).

Reperfusion, Ca^{2+} and stunning. The net gain in tissue Ca^{2+} on abrupt reperfusion has been known for many years (Jennings and Shen, 1972). However, despite the advances in technology, conclusive data concerning cytosolic Ca^{2+} concentrations during ***early*** reperfusion and its involvement in reversible reperfusion injury has been elusive.

The limited success achieved in measuring cytosolic Ca^{2+} levels on reperfusion has been due to the slow response time of the cytosolic Ca^{2+} measuring methods

presently available to researchers. If the concept that reperfusion may induce a transient increase in cytosolic Ca^{2+} in the reversibly injured myocardium is correct, then measurements would have to be performed at short intervals throughout early reperfusion (within the first 2 minutes). Marban and co-workers (1989), and Steenbergen and co-workers (1987) measured intracellular Ca^{2+} concentrations during reperfusion but were unable to measure the cytosolic Ca^{2+} concentrations within the first 2.5 minutes of reperfusion. By the time the first Ca^{2+} measurement was made after 2.5 minutes of reperfusion, intracellular Ca^{2+} levels had normalised (Marban *et al.*, 1989). In 1990, Marban and co-workers performed another study measuring reperfusion Ca^{2+} levels during early reperfusion. They documented a persistent elevation in the reperfusion cytosolic Ca^{2+} levels. This prolonged elevation of intracellular Ca^{2+} levels during reperfusion was however only evident when perfusate Ca^{2+} concentration was 8 mM (to compensate for the BAPTA induced buffering of Ca^{2+}) and hearts were paced throughout ischaemia (causing severe ischaemia) and reperfusion. In another study, simulated ischaemia of 45 minutes caused a large prolonged increase in cytosolic Ca^{2+} levels and mechanical failure in isolated ferret papillary muscle preparation (Lee and Allen, 1992). This study was however dealing with simulated ischaemia and a long "ischaemic" period which caused reperfusion mechanical failure and does not reflect a model of true reversible reperfusion injury. There is thus far no direct evidence to show that cytosolic Ca^{2+} levels are elevated during the first couple of seconds or minutes of reperfusion in the reversibly injured myocardium.

The most direct evidence to support the role of transient Ca^{2+} overload in inducing stunning was the observation that it (in the absence of ischaemia) induced mechanical and metabolic abnormalities similar to those seen with stunning (Kitikaze, Weisman and Marban, 1988). Also, electrically induced

ventricular fibrillation, which is associated with increased cytosolic Ca^{2+} levels (Kihara and Morgan, 1991), causes contractile dysfunction (Koretsune and Marban, 1989).

2.6 Ischaemia, Ca^{2+} and Ventricular Arrhythmias.

Historically, the earliest hypothesis to explain the ionic basis of ventricular arrhythmias was ischaemic potassium loss (Harris *et al.*, 1954; Hirche *et al.*, 1980). Later, the role of sympathetic nerve activity and its messenger, cyclic AMP was investigated (Podzuweit, Lubbe and Opie, 1976; Lubbe *et al.*, 1978) and in the past two decades, increased cytosolic Ca^{2+} concentrations have been associated with, and implicated in the genesis of arrhythmias (Clusin 1983; 1989; Lee *et al.*, 1988; Koretsune and Marban, 1989; Merillat *et al.*, 1990; Kihara and Morgan 1991; Kusuoka, Chacko and Marban, 1992).

Although ischaemic K^+ loss is still linked to the genesis of arrhythmias, the cyclic AMP theory has been somewhat controversial. It has been found that forskolin, which increases cyclic AMP levels in the rat heart, does not increase the incidence of ventricular fibrillation (Manning *et al.*, 1985). These data are further supported by data showing that metoprolol (a ß-blocker) decreased the incidence of ischaemic ventricular fibrillation without having an effect on the cyclic AMP levels in the ischaemic myocardium (Muller *et al.*, 1986). Despite this contradictory evidence showing that there is no strict correlation between the incidence of arrhythmias and increases in cAMP levels, it is believed that a correlation between the levels of this messenger, elevated cytosolic Ca^{2+}, and arrhythmias does exist (Lubbe, Podzuweit and Opie, 1992). The two ischaemia induced ionic changes that are still thought to play a major role in the genesis of ventricular arrhythmias are, 1) an ischaemia induced K^+ loss, and, 2) an ischaemia and reperfusion induced intracellular Ca^{2+} gain. Data presently

available suggests that an elevated cytosolic Ca^{2+} concentration is the final common path of several arrhythmogenic mechanisms including those related to sympathetic nerve activity and elevated cAMP levels.

Electrophysiological evidence for the role of Ca^{2+} in arrhythmogenesis. In the early 1970s, Ferrier, Saunders and Mendez (1973) studied the effects of strophanthidin (digitalis) on the action potential of the isolated canine Purkinje fibre (Figure 2.7 illustrates two possible modes of action of digitalis on the fibre to increase Ca^{2+}). They noticed that after cessation of a train of 10 stimuli, strophanthidin superfusion of the fibre induced an electrophysiological manifestation which was called a transient afterpotential (Figure 2.8). They concluded that these transient afterpotentials could be the underlying mechanism for digitalis-induced automaticity by achieving threshold potential and initiating extrasystoles.

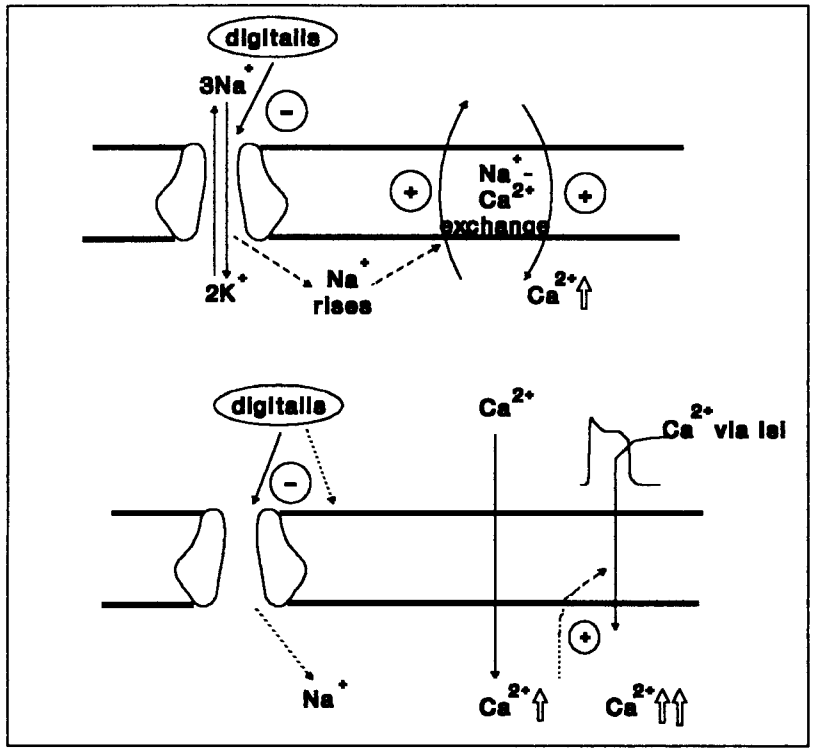

FIGURE 2.7 *Two possible modes of action of digitalis (strophanthidin) in increasing internal Ca^{2+} concentration. By inhibiting the Na^{+},K^{+}-ATP-ase pump, digitalis poisoning may promote intracellular Na^{+} loading, Na^{+}/Ca^{2+} exchange activation and finally, cytosolic Ca^{2+} loading. Alternatively, digitalis may act on its receptor, and promote (mechanism unknown) Ca^{2+} influx across the sarcolemma (figure reproduced from Opie, 1986).*

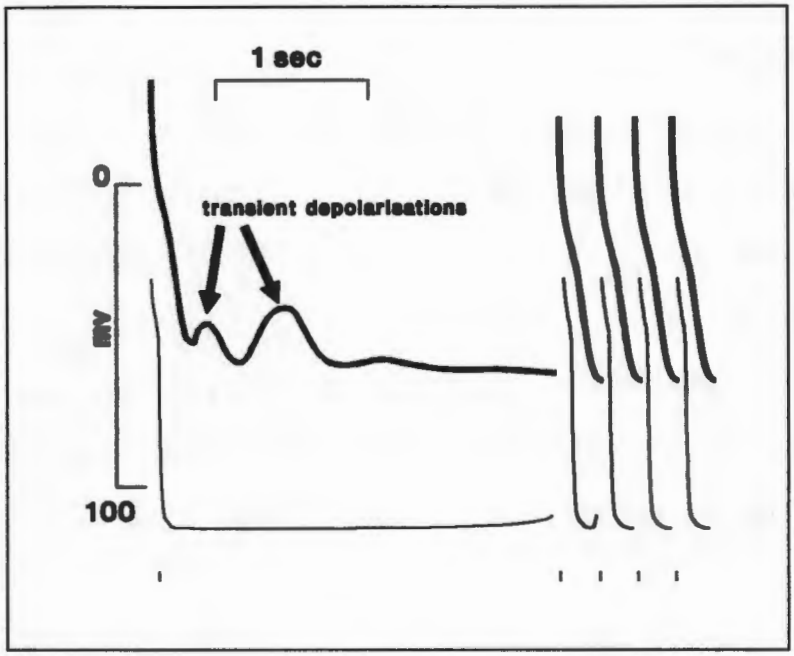

FIGURE 2.8 *Effect of acetylstrophanthidin ($1X10^{-7}g/ml$) on the transmembrane potentials of Purkinje fibres (top trace) and muscle (bottom trace). The first action potential is the last of a train of ten driven potentials. During the pause in stimulation, two transient depolarisations (or DADs) coupled to the last action potential occur in the Purkinje fibre but not in the muscle (figure reproduced from Ferrier, Saunders and Mendez, 1973).*

Transient depolarisations (afterpotentials) were subsequently given the more appropriate name of oscillatory afterpotentials (OAP) and it was determined that Ca^{2+} played an important role in the genesis of OAPs as the amplitude of these OAPs was directly related to the Ca^{2+} concentration of the superfusion solution (Ferrier and Moe, 1973). Removal of extracellular Ca^{2+} or the use of $MnCl_2$ (manganese is a Ca^{2+} channel blocker) quickly and reversibly abolished OAPs. In this study, they (Ferrier and Moe, 1973) suggested that the OAPs may be caused by a transient inward current carried by Ca^{2+}. Shortly after these observations were made, a strophanthidin induced oscillatory inward current (TI) was linked to OAPs (Lederer and Tsien, 1975).

In 1985, when Ferrier, Moffat and Lukas did electrophysiological studies on isolated canine Purkinje fibre-papillary muscle preparations, they documented an ischaemia induced loss of membrane potential, shortening of action potential, decreased excitability, and spontaneous electrical activity. With reperfusion, there was an increase in the incidence of oscillatory afterpotentials and automaticity (and susceptibility to arrhythmias) of these papillary muscles which was thought to be a direct consequence of the electrophysiological changes seen during ischaemia. The term OAP subsequently evolved further and is at present better known as the delayed afterdepolarisation (DAD) (Ferrier, 1977).

Work in our laboratory has shown that DADs could be evoked by, fast pacing, isoproterenol, cAMP and intracellular Ca^{2+} injections (Coetzee and Opie, 1987). The factor common to these stimuli is an elevated cytosolic Ca^{2+} concentration. These DADs could however not be evoked during severe ischaemia (Coetzee and Opie, 1987; Opie and Coetzee, 1988). Based on these findings, they proposed that at least some ATP was necessary for Ca^{2+} dependent arrhythmias to occur and that these Ca^{2+} induced arrhythmias are more likely to occur during early or mild ischaemia, or during reperfusion when ATP levels are high enough to promote and maintain intracellular Ca^{2+} oscillations. With reperfusion after severe ischaemia, ATP may be regenerated and may become available to the SR Ca^{2+} ATP-ase pump for oscillatory intracellular Ca^{2+} cycling (Coetzee *et al.*, 1988).

The mechanism underlying DADs is the Ca^{2+} dependent transient inward current (I_{ti})(Figure 2.9). It is proposed that the rhythmic internal Ca^{2+} oscillations cause rhythmic switching on and off of the transient inward current which presents as delayed afterdepolarisations after the normal action potential.

In ventricular muscle, delayed afterdepolarisations are a gross electrophysiological abnormality which can lead to triggered automaticity and hence play a role in converting a focus of automaticity into sustained ventricular arrhythmias.

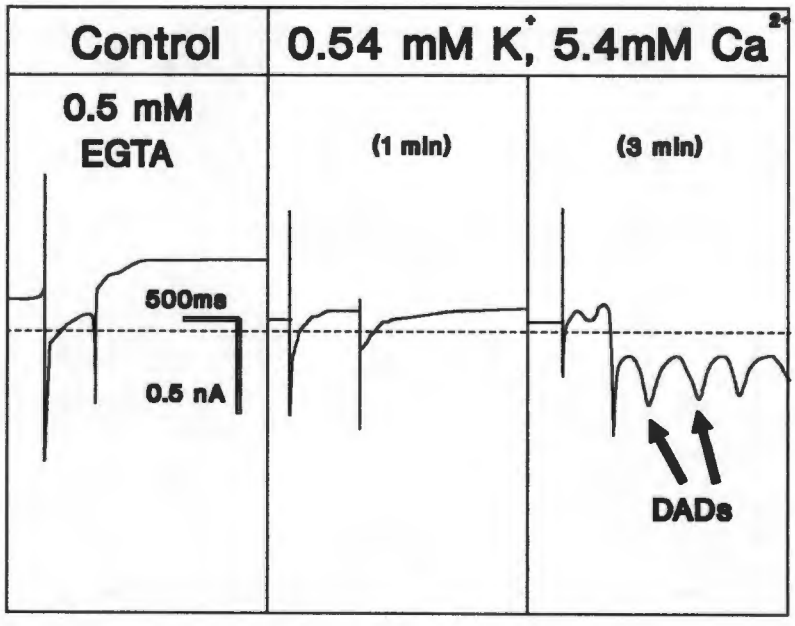

FIGURE 2.9 *Ventricular myocytes were voltage clamped from a holding potential of -55 mV to a test potential of -5 mV using rectangular voltage pulses with a duration of 500 msec. When the pipette filling solution contained 0.5 mM EGTA, the I_{ti} was elicited within 3 minutes after changing over to an extracellular solution of 0.54 mM K^+ and 5.4 mM Ca^{2+} (reproduced from action potential traces recorded by Dr WA Coetzee and published in Opie and Coetzee, 1990)).*

Later studies showed beyond doubt that delayed afterdepolarisations are associated with reperfusion arrhythmias (Priori *et al.*, 1988), and that neither delayed afterdepolarisation, nor the transient inward current occurs without intracellular Ca^{2+} oscillations (Berlin, Cannell and Lederer, 1989; Boutjir, El-Sherif and Gough, 1990). It has also been found that both caffeine and ryanodine

given during ischaemia in the dog model decreased the incidence of DADs and triggered activity (Boutjir, El-Sherif and Gough, 1990), providing further evidence to show that DADs are related to intracellular Ca^{2+} oscillations between the SR and the cytosol. Figure 2.10 is a diagrammatic summary of the potential mechanisms by which the Ca^{2+} current could be arrhythmogenic.

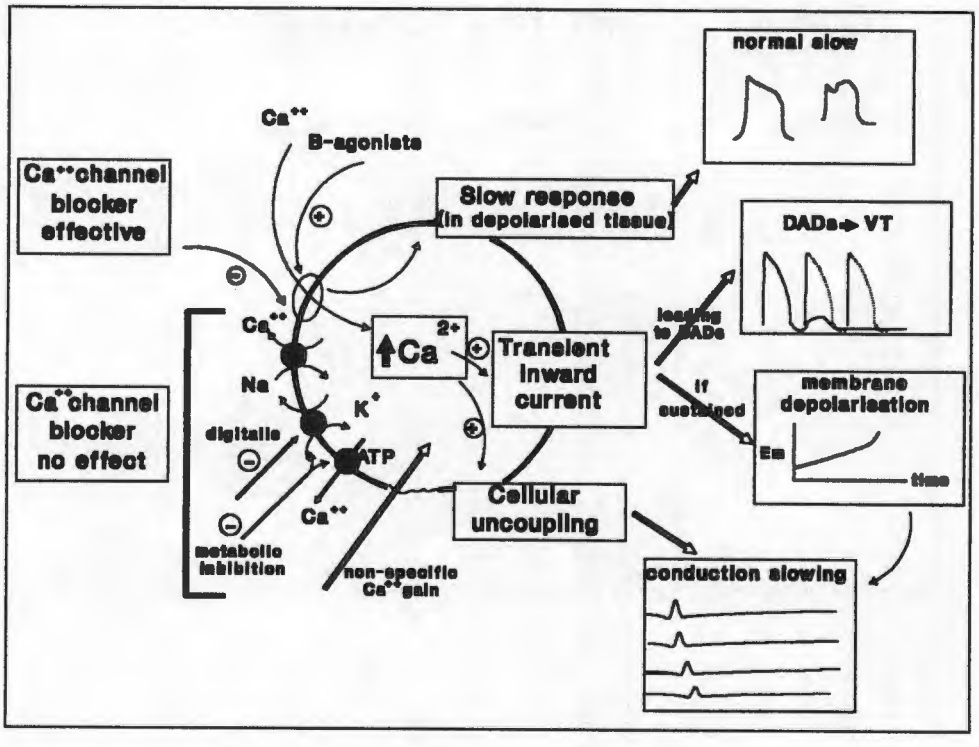

FIGURE 2.10 *Cell Ca^{2+} and arrhythmias. Assuming that intracellular Ca^{2+} does increase during ischaemia, there are a number of potential mechanisms by which the Ca^{2+} current could be arrhythmogenic: (a) Ca^{2+} influx might elicit slow responses; (b) an additional increase in cytosolic Ca^{2+} could activate oscillatory inward current flow and precipitate DADs; (c) if the Ca^{2+} activation of this inward current were sustained, then accelerated depolarisation might increase injury current across the ischaemic boundary and precipitate fibrillation; (d) through promoting depolarisation and increasing the coupling resistance between cells, a raised cytosolic [Ca^{2+}] might also slow conduction and increase the likelihood of reentry. Ca^{2+} entry blockers should be effective against the effects of a gain of intracellular Ca^{2+} resulting from enhanced Ca^{2+} entry through the Ca^{2+} channels (figure reproduced from Opie et al., 1988).*

Indirect evidence for the role of intracellular Ca^{2+} oscillations in arrhythmogenesis. In a study investigating the possible role of abnormal cytosolic Ca^{2+} levels in the genesis of arrhythmias, it was seen that acidosis, in the absence of ischaemia could induce spontaneous Ca^{2+} release (and thus diastolic Ca^{2+} oscillations) from the SR in isolated papillary muscles (Orchard *et al.*, 1987). This group concluded that ischaemia induced acidosis could increase the spontaneous release of Ca^{2+} from the Ca^{2+} loaded SR, increase diastolic Ca^{2+} oscillations and the incidence of spontaneous action potentials, and may thus promote arrhythmias in the ischaemic heart. Data obtained with the isolated ferret papillary muscle under conditions of simulated ischaemia, also show that an increase in the incidence of spontaneous cytosolic Ca^{2+} oscillations corresponds well with the period in ischaemia during which ischaemic arrhythmias are most frequent (Lee and Allen, 1992).

Indirect evidence from our laboratory also suggests that abnormal intracellular Ca^{2+} oscillations between the sarcoplasmic reticulum and the cytosol may play a role in the genesis and perpetuation of both ischaemic and reperfusion arrhythmias (Figure 2.11). Thandroyen and co-workers (1988), showed that both ryanodine and caffeine could decrease the incidence of arrhythmias in the rat heart model. These compounds, known to act on the SR Ca^{2+} release channel to prevent Ca^{2+} release from the sarcoplasmic reticulum may decrease or eliminate these oscillations that may play a role in the initiation or perpetuation of Ca^{2+} induced arrhythmias.

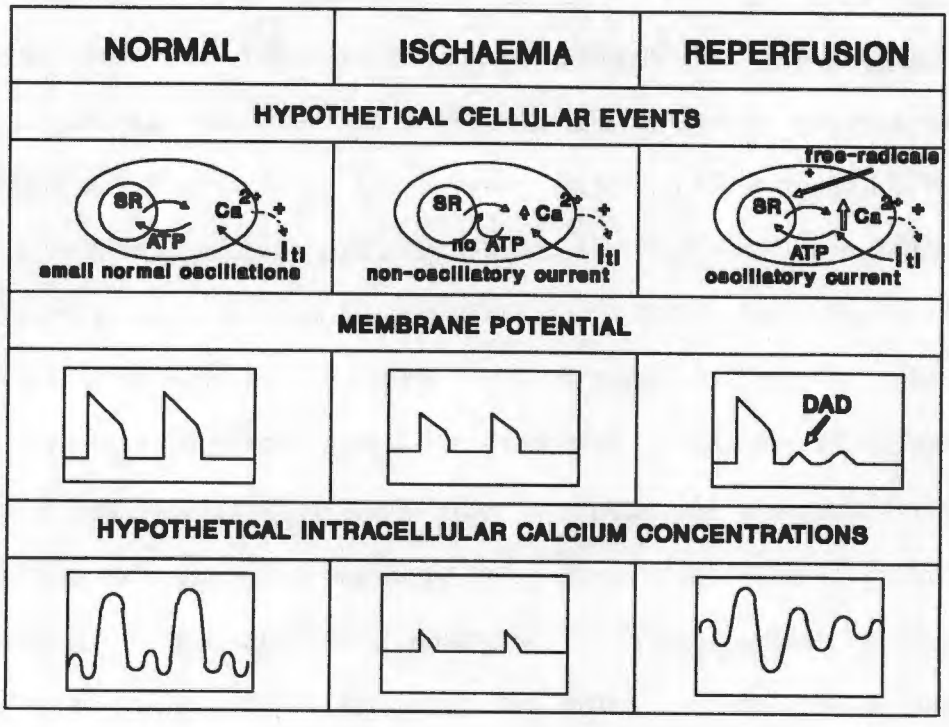

FIGURE 2.11 *A possible sequence of events occurring in normal, ischaemic, or reperfused myocardial cells. In each case, a schematic representation of the myocardial cell and the sarcoplasmic reticulum is shown. In the bottom panel, action potentials are illustrated along with the hypothetical changes in the intracellular Ca^{2+} concentrations. Note that in severe ischaemia, although the internal cytosolic Ca^{2+} rises, Ca^{2+} flux in and out of the sarcoplasmic reticulum is inhibited, and therefore oscillatory I_{ti} cannot develop. On the other hand, sustained inflow of current via I_{ti} leads to increased depolarisation, which enhances the difference between the ischaemic and non-ischaemic zones and predisposes to arrhythmias. With reperfusion, when ATP becomes available, and Ca^{2+} fluxes in and out of the sarcoplasmic reticulum are resumed, an oscillatory I_{ti} can develop at the lower postulated intracellular Ca^{2+} concentration and arrhythmias dependent on DADs may be provoked (figure reproduced from Opie et al., 1988).*

More recently, Thandroyen and co-workers (1991), investigated the effect of elevated extracellular Ca^{2+} concentrations in the perfusate on intracellular Ca^{2+} levels in isolated myocytes, and found that elevated extracellular Ca^{2+} concentrations caused an elevation in intracellular Ca^{2+} and lead to

spontaneous Ca^{2+} oscillations during diastole (Thandroyen *et al.*, 1991). These spontaneous oscillation were characteristic before the initiation of spontaneous arrhythmia-like activity in these cells. In these cells, ryanodine could inhibit membrane potential oscillations that were characteristic of the transient inward current. This data was substantiated by Kihara and Morgan (1991) who measured intracellular Ca^{2+} transients before and during electrically induced ventricular fibrillation and found that increased diastolic Ca^{2+} transients (oscillations) preceded the initiation of ventricular fibrillation. Also, ryanodine could decrease these oscillations and the susceptibility of the heart to ventricular fibrillation. Thus, not only elevated cytosolic Ca^{2+} levels, but also augmented Ca^{2+} oscillations may contribute to the initiation and perpetuation of certain types of arrhythmias.

A role for free radicals in the genesis of ischaemic and reperfusion arrhythmias was proposed by Manning and co-workers (1984; 1988) who showed that allopurinol (xanthine oxidase inhibitor) decreased the incidence of reperfusion arrhythmias from 67 to 11%. Although various subsequent studies have confirmed this data and presented conclusive evidence for the role of free-radicals in arrhythmogenesis (Riva, Manning and Hearse, 1987; Bolli *et al.*, 1988b; Bolli *et al.*, 1989; Bernier, Manning and Hearse, 1986; Hearse and Tosaki, 1987) this aspect of reperfusion injury falls beyond the scope of this study and will not be discussed in detail.

CHAPTER 3

HYPOTHESIS UNDER TEST.

3.1 Evolution of Concepts in our Laboratory Leading to the Hypothesis that Ca^{2+} Contributes to Arrhythmogenesis and Stunning.

After early observation indicating that catecholamine stimulation increased the vulnerability of the heart to ventricular fibrillation (Hoffman *et al.*, 1955), and those linking coronary artery ligation to activation of the adrenergic system, (Karlsberg *et al.*, 1979), researchers in our laboratory performed extensive studies aimed at elucidating the possible mechanisms causing myocardial ischaemic and reperfusion arrhythmias. At this stage, several studies showed that administration of norepinephrine could elicit arrhythmias (Lubbe *et al.*, 1978; Opie *et al.*, 1980; Podzuweit, 1980), while beta-receptor blockade administration protected against arrhythmias (Muller *et al.*, 1986) in the ischaemic heart in a number of animal models.

In 1978 Podzuweit and co-workers linked the ischaemic elevation of cAMP to ventricular fibrillation. Subsequently, various researchers in our laboratory studied the effects of acute regional myocardial ischaemia on the baboon and isolated rat heart model (Podzuweit, Lubbe and Opie, 1976; Lubbe *et al.*, 1978; Opie, Nathan and Lubbe, 1979) from which the cyclic AMP hypothesis evolved. According to this hypothesis, cyclic AMP, the second messenger of the adrenergic system, plays a key role in the mediation of the adrenergic effects on vulnerability to fibrillation.

This hypothesis was based on the following: i) Podzuweit and co-workers, 1978, and others (Opie, Nathan and Lubbe, 1979) noted an increase in cAMP levels prior to the development of ventricular fibrillation in the baboon and pig model respectively, and ii) Lubbe and co-workers (1979, 1981, 1983) found that

suppressing the increases in ischaemic cAMP levels with propranolol, amiodarone or labetalol prevented the ischaemia induced decrease in ventricular fibrillation threshold in the isolated rat heart model.

After a relation between cellular levels of cAMP, Ca^{2+} mediated inward current and electrical excitability had been established (Watanabe and Berch, 1974), it was proposed that Ca^{2+} may be involved in arrhythmogenesis. Thandroyen, (1982) and Lubbe, McLean and Nguyen (1983) started investigating the possible protective effects of Ca^{2+} antagonists against arrhythmias and found that verapamil prevented a decrease in the ventricular fibrillation threshold in the isolated rat heart model.

Electrophysiological evidence for the role of Ca^{2+}: The next series of studies performed in our laboratory were electrophysiologic and were aimed at trying to establish whether a Ca^{2+} current caused delayed afterdepolarisations (DADs) as proposed by Janse (1980). Saman, Coetzee and Opie (1988) found that DADs could be exacerbated by dibutyryl cAMP and isoproterenol under control conditions but not during ischaemia in isolated papillary muscle preparation. DADs were again precipitated on reperfusion, which was interpreted as an indication that DADs are ATP dependent and only occur during mild ischaemia or during reperfusion (Coetzee and Opie, 1987).

After these successful studies investigating the possible role of Ca^{2+} in ischaemic and reperfusion arrhythmias, Coetzee and co-workers (1990) investigated the possible role of oxygen derived free radical in reperfusion arrhythmias. Their data using the isolated guinea pig papillary muscle, showed that the time course of electrophysiological changes induced by free-radical generating systems are slow. Based on this data showing that free-radical

generating and scavenging systems, when used in their preparation, had no effect on arrhythmias, they concluded that it is unlikely that free-radicals are a prime cause of early reperfusion arrhythmias. They felt that the mechanism of early reperfusion arrhythmias was more likely to be a Ca^{2+} sensitive process with free-radicals mediating delayed membrane changes.

These studies presented good evidence for a role for Ca^{2+} but not free-radicals in the genesis of reperfusion arrhythmias. We subsequently proposed that abnormal Ca^{2+} fluxes at the onset of reperfusion may, in conjunction with oxygen derived free radicals contribute to reperfusion stunning and arrhythmias.

3.2 Aim of the Study.

The purpose of this study was to obtain a better understanding of the role of Ca^{2+} and the sources contributing to ischaemic and reperfusion Ca^{2+} overload and reperfusion injury. Although measurements of intracellular Ca^{2+} levels would indicate whether Ca^{2+} levels are elevated during reperfusion, the slow response time of present Ca^{2+} measuring techniques prevents one from measuring intracellular Ca^{2+} levels from the onset of reperfusion. Also, these measurements would not elucidate the origin, or the entry pathways which contribute to this increase in cytosolic Ca^{2+} levels. For our investigation looking at the possible contribution of transient intracellular Ca^{2+} overload to reperfusion injury, we therefore chose to manipulate cellular Ca^{2+} fluxes with pharmacological compounds specific to certain channels, pumps and exchangers. These interventions were usually made before ischaemia, or during reperfusion in an attempt to modulate transsarcolemmal and intracellular Ca^{2+} fluxes during ischaemia or at the onset of reperfusion.

In order to achieve this, we endeavoured to manipulate ischaemic and reperfusion induced intracellular and transsarcolemmal Ca^{2+} fluxes by:

1) Altering reperfusion transsarcolemmal Ca^{2+} fluxes with a) ß-receptor stimulants, Ca^{2+} channel agonists, Ca^{2+} channel antagonists or an endogenous peptide, endothelin, or b) indirectly, by Na^{+}/H^{+} exchange inhibition.

2) Altering intracellular Ca^{2+} fluxes between the sarcoplasmic reticulum (SR) and cytosol with SR Ca^{2+} release channel blockers or, SR Ca^{2+} ATP-ase pump inhibitors (Figure 3.1).

The end points to these studies were the severity of reperfusion stunning, the incidence of ventricular arrhythmias, and various biochemical parameters which will be described and discussed in the following chapters.

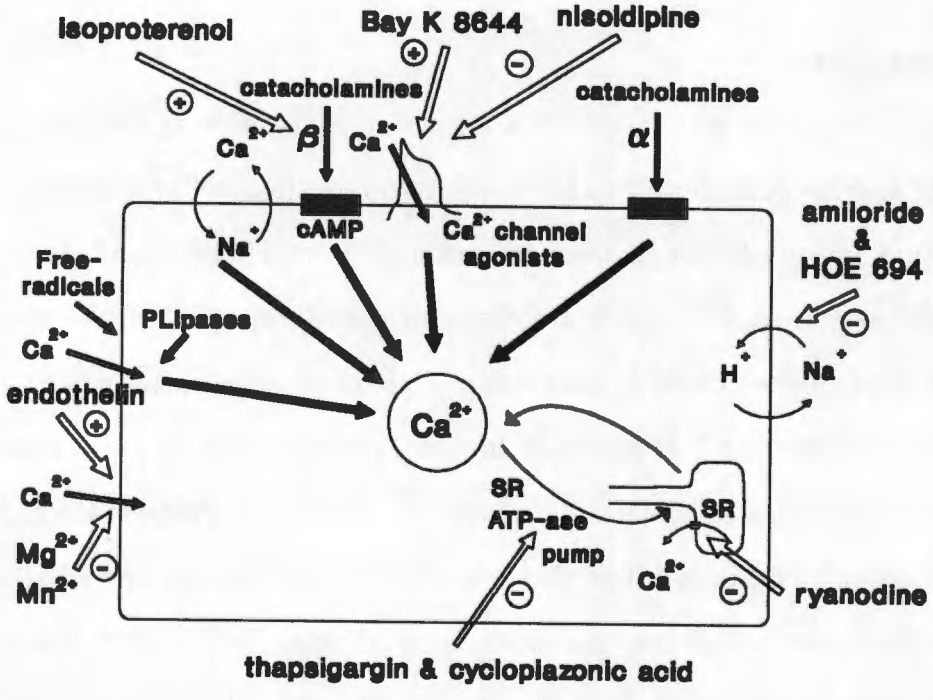

FIGURE 3.1 *Diagrammatic representation of the cell, the pharmacologic compounds used, and their mode of action on the respective channels, pumps and exchangers in the heart.*

CHAPTER 4

EXPERIMENTAL MODEL, MATERIALS AND METHODS

4.0 The Isolated Rat Heart Model.

The isolated rat heart model was used to study the effects of interventions aimed at modulating Ca^{2+} fluxes during ischaemia and/or at the onset of reperfusion on the severity of reperfusion stunning and the incidence of arrhythmias after regional or global ischaemia. The apparatus used was first described by Langendorff (1895), and later modified by Neely and co-workers, (1967) (Figure 4.1). The system consists of two parts; the Langendorff perfusion side, used to perfuse the heart in a retrograde fashion, and the working heart side used to perfuse the heart in the working heart mode.

Animal used: Male Long-Evans rats weighing 250-350 grams and fed a standard rat chow diet were used for all experiments performed in this study. All animals received care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by National Institutes of Health (NIM Publication No. 80-23, revised 1978).

i) Langendorff model.

Rats were anaesthetised by diethyl ether inhalation, heparinised with 200 IU of heparin injected into the femoral vein, and the hearts were removed rapidly and plunged in cold ($4^{o}C$) Krebs-Henseleit buffer for transfer to the perfusion apparatus. Arrest was induced to afford the hearts maximal protection during the brief ischaemic episode during transfer to the perfusion apparatus. The aorta was cannulated and perfusion re-established within 1 minute of excision from the donor animal. Normothermic Krebs-Henseleit buffer was oxygenated with 95%

O_2 and 5% CO_2 (to a pO_2 of more than 550mmHg) and filtered through cellulose filters (0.8μm pore size), before being allowed to flow retrogradely into the aorta at a pressure of 100 cm$H_2$0. The pulmonary artery was incised to allow free drainage of the coronary effluent. Hearts were maintained at 37^{o}C throughout the experiment by surrounding them with a water jacketed chamber maintained at this temperature. The Langendorff perfusion apparatus was used; 1) to allow the heart to stabilise at the beginning of the experiment and to initiate reperfusion of the heart after ischaemia and, 2) when regional ischaemia was induced to promote ischaemic and reperfusion arrhythmias during the arrhythmia studies.

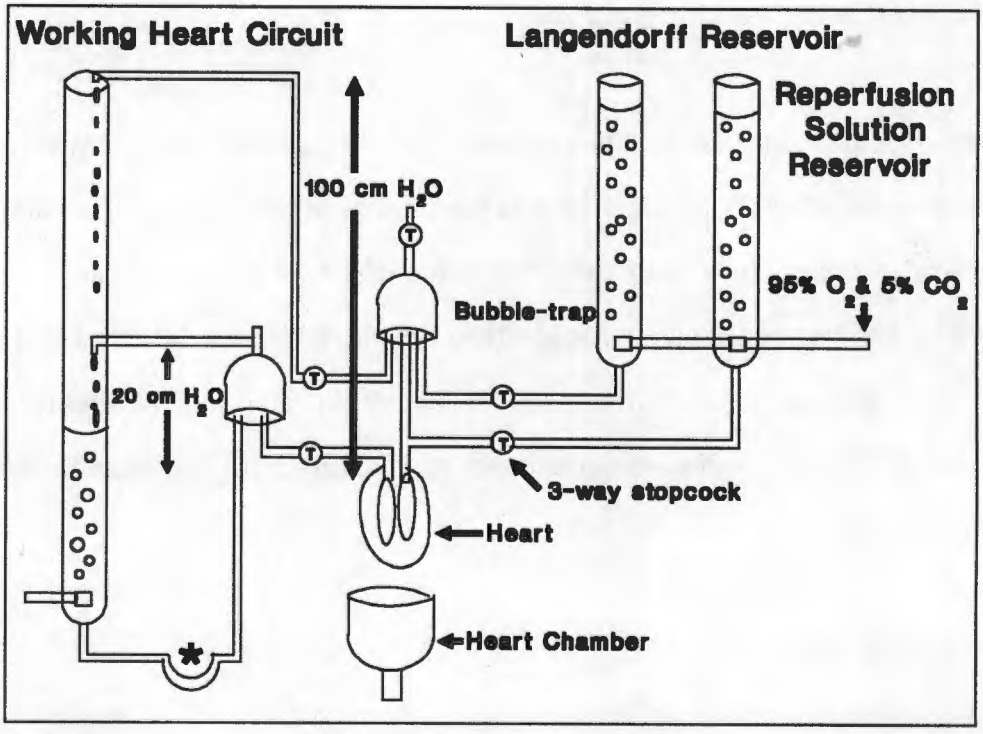

FIGURE 4.1. A diagrammatic representation of the Langendorff and working heart preparation used in this study.

ii) Working heart model.

In order to measure mechanical functional and metabolic recoveries of the heart and evaluate the severity of reperfusion stunning, the working heart model was

used. For the working heart preparation, the left atrium and aorta were cannulated and atrial perfusion (the preload) was maintained at a perfusion pressure of 20 cmH_2O while the afterload was 100 cmH_2O. Perfusate temperature was again maintained at 37^oC.

Under normal working heart conditions, the left atrium ejects the perfusate to the ventricle which then pumps the crystalloid perfusate through the aortic cannula.

To monitor left ventricular pressure, the left ventricle was cannulated using Abbocath 20G drip administration needles (Abbott, Kent, UK.). To the needle was attached a Stratham Gould P23ID pressure transducer and a Lectromed Multitrace 2 chart recorder (Lectromed Limited, Rue Fondon, St Peter, Jersey Channel Islands, USA.) to monitor the pressure throughout the experiment.

In the Langendorff perfused rat heart, mechanical function was evaluated using a left ventricular "Clingfilm" balloon. The balloon was inserted into the left ventricle via the atrium, inflated to a diastolic pressure of 4-8 mmHg and attached to the Stratham Gould P23ID pressure transducer and Lectromed chart recorder.

Unless otherwise specified, hearts were paced at 5 Hz (300 bpm) for the entire experiment except during ischaemia and the first 2 minutes of reperfusion using a Grass S88 stimulator (Grass Medical, Quincy, Massachusetts, USA) and two platinum pacing electrodes placed at the base of the left ventricle. Pacing from the onset of reperfusion invariably induced reperfusion tachycardia and/or fibrillation and early pacing was therefore avoided. Pacing pulse width was maintained at 0.2 msec, and voltage at 2-5 volts.

For all experiments performed to determine functional parameters before and after ischaemia, left ventricular pressure, aortic flow, coronary flow and cardiac output was determined and compared with pre-ischaemic values. Aortic output and coronary flow were measured manually.

4.1 Exclusion Criteria: failed experiments.

In the pre-ischaemic control period, hearts were evaluated and those not satisfying certain minimum requirements, were excluded. These were:-

Aortic outputs < 40 ml/min.

Coronary flows > 22 ml/min.

Heart rates below 200 beats/min., or irregular rhythm.

In the post-ischaemic reperfusion period, hearts that had coronary flow rates higher than 50% of the aortic output, were excluded. These high values were normally indicative of a left atrial or an aortic leak.

4.2 Perfusion Solution Used.

Standard perfusion solution: A modified Krebs-Henseleit buffer (mmol/l NaCl-118.5, $NaHCO_3$-25, KCl-4.75, KH_2PO_4-1.18, $MgSO_4$-1.19, $CaCl_2$-1.25, glucose-11), aerated with a carbogen mixture of 95 %O_2 and 5 %CO_2 was used to perfuse the hearts at 37°C. The buffer was made up from stocks solutions daily. To avoid precipitation of the Ca^{2+}, all solutions were first aerated with the carbogen mixture before the $CaCl_2$ was added. Solutions were filtered through 5.0 μm and 0.8 μm (Millipore Corporation, Bedford, MA, USA) cellulose filters before use.

Modified solutions used during early reperfusion. When reperfusion solutions contained high cation concentrations which tended to precipitate with bicarbonate, a modified Hepes buffered Tyrode's solution was used to replace the Krebs-Henseleit buffer. (mmol/L NaCl 137, KCl 5.4, Hepes buffer 10, $MgCl_2$ 0.5, $CaCl_2$ 1.25, and glucose 10). This solution was gassed with 100% O_2.

4.3 Experimental Protocol used for Reperfusion Mechanical Function Studies.

For all experiments performed to determine reperfusion function after brief ischaemia, the same protocol was employed.

Hearts were mounted on the perfusion apparatus, allowed a 10 minute equilibration period in the Langendorff mode and then made to work in the working heart mode. Hearts were made to work for 10 minutes and function measured at 5 and 10 minutes. Hearts subjected to pre-ischaemic drug treatment were treated in the Langendorff mode for 2-5 minutes before being made globally ischaemic for 20 minutes at 35-37^{o}C. Hearts subjected to the drug at reperfusion were reperfused with the drug in the Langendorff mode for 2 minutes after which drug infusion was stopped and pacing engaged for 3 minutes before the working heart mode was again employed (Figure 4.2). Hearts were made to work for 10-30 minutes after switching to the working heart mode, and mechanical function was monitored.

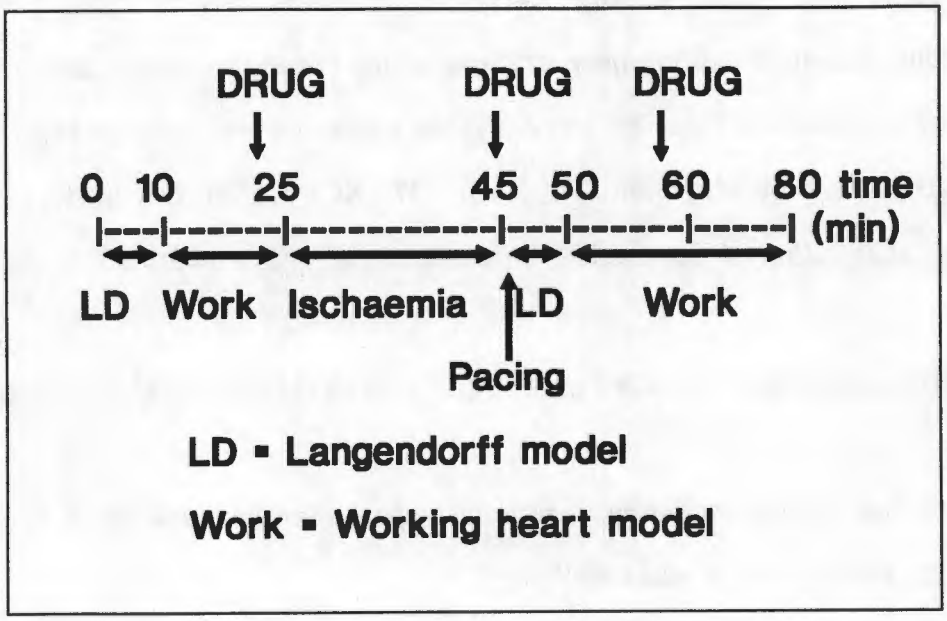

FIGURE 4.2 *Experimental protocol used during studies for this thesis. Interventions were made before ischaemia (25 minutes), at the onset of reperfusion (45 minutes) or late in reperfusion (60 minutes).*

Model for induction of regional ischaemia in arrhythmia studies.

For studies in which ischaemic and reperfusion arrhythmias were to be induced and documented, a model of regional ischaemia was employed. The left anterior descending coronary artery was ligated using a 3-0 Ti-cron suture (Davis & Geck, Wayne, NJ, USA). This procedure reduced coronary flow by a minimum of 25 % and about 35 % of the left ventricle was rendered ischaemic (Lubbe, Daries and Opie, 1978). Electrocardiograph (ECG) electrodes were attached to the aortic cannula and the right ventricle and the incidence of ischaemic and reperfusion arrhythmias were monitored by means of an electrocardiogram on a Lectromed Multitrace 2 chart recorder.

4.4 Parameters Evaluated.

To evaluate reperfusion recovery, mechanical, metabolic and morphological parameters were measured. In this study, mechanical function recovery was the most important parameter to evaluate the severity of reperfusion injury.

A. Haemodynamic parameters.

Because the majority of experiments were performed using paced hearts, the heart rate was consistent and thus had no effect on cardiac output (CO) or aortic outputs (AO) values.

Coronary flow (CF) was measured and used in conjunction with aortic output to give the CO values.

$CO = AO + CF.$

Left ventricular developed pressure (LVDP) was measured using the left ventricular cannula in the working heart preparation, and the left ventricular balloon in the Langendorff preparation. LVDP was obtained by subtracting the diastolic pressure from the systolic pressure.

LVDP = systolic pressure - diastolic pressure.

Values (AO or CO) obtained in the post-ischaemic working period were expressed as a percentage of their individual pre-ischaemic values and presented as a % recovery.

$$\% \text{ AO or CO recovery} = \frac{\text{reperfusion AO or CO} \times 100}{\text{pre-ischaemic AO or CO}}$$

In all cases, drug treated groups of hearts were performed with a concurrent control group for comparative purposes.

B. Metabolic parameters.

Tissue extraction procedure: Hearts were freeze clamped with Wollenberger clamps pre-cooled in liquid nitrogen and freeze dried for 24 hours in a Christ 1-4 freeze drier (Osterode/Harz, Germany). Freeze dried tissue (20 mg) was added to 1.2 ml 6% perchloric acid and homogenised for approximately 20 sec (while on ice) with a Polytron homogeniser (Luzern, Switzerland). The extracts were then spun down in a Sigma 202MK centrifuge at 5000 RPM and 4^{o}C for 10 minutes, and the supernatant separated and neutralised with Tris/KOH buffer to a pH of 7.0. Universal indicator was used to determine the neutral pH. The sample was again spun at 5000 rpm and the supernatant removed. The sample was now ready for cAMP, ATP and PCr assays. All compounds were assayed enzymatically by spectrophotometry or with the use of radioimmunoassay kit. Standards, internal standards and blanks were included in every assay.

i) ATP and CP (PCr).

The ATP and CP of samples from heart tissue were determined according to the method of Lamprecht and Trautschold (1963) based on the breakdown of glucose to form glucose-6-phosphate (G-6-P).

$$\text{CP} + \text{ADP} \xrightarrow{\text{Creatine phosphokinase}} \text{ATP} + \text{creatine}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{Hexokinase}} \text{ADP} + \text{G 6-P}$$

$$\text{G 6-P} + \text{NADP} \xrightarrow{\text{Glucose 6-phosphate dehydrogenase}} \text{6 PGA} + \text{NADPH}^{+} + \bar{\text{H}}^{+}$$

The following cocktail was used:

	Stock	Cocktail (per cuvette)
$MgCl_2$	1 mol/l	0.1 ml
Tris	0.2 mol/l	1.0 ml
NADP	1% (w/v)	0.1 ml
Glucose	100 mmol/l	0.05 ml
Dist H_2O		1.55 ml
Sample		0.2 ml

Enzymes used were:
G6PDH 1 mg/ml
HK 10 mg/ml
ADP 10mM
CK 10mg/ml

0.2 ml sample and 5 μl G6PDH was added to 2.8 ml cocktail per cuvette. The extinction at 340 nm was read with a Beckman DU-7 Spectrophotometer (Beckman Instruments Inc., Irvine, California). 5 μl hexokinase was added to each cuvette, and the extinction at 340 nm read after 10 minutes when stable.

The difference in optical density was obtained and used to calculate ATP concentration.

(δ OD - blank)/0.414 * F = μmol/gm dried weight.

F = the dilution factor taken into consideration with the neutralising of the Tris buffer.

$$F=[1.2ml(PCA) \times \text{total volume}]/\text{wt (g)}$$

Total volume = neutralising volume + 5 μl Universal indicator.

For the determination of CP (PCr) levels, 0.05 ml of ADP was added to the cuvette. After 10 minutes, a reading was taken at 340 nm. This was taken as the

baseline reading of PCr. CK was added the cuvette allowed to stand for 20-30 minutes and the absorbance read. PCr values were again calculated using the formula given above.

The tissue high energy phosphate levels were expressed as μmol/gm fresh weight. Values obtained for freeze-dried tissue were divided by a factor of 5 to obtain their wet weights.

Although high-performance liquid chromatography has gained favour over enzymatic techniques for the determination of tissue high energy phosphate levels, we chose to use enzymatic methods in this study. Previous published (Victor *et al.*, 1987) and unpublished data from our laboratory has shown that high energy phosphate levels obtained using these two measuring techniques are highly comparable.

ii) Cyclic AMP.

Cyclic AMP (cAMP) was measured by the method described by Tovey and co-workers (1974). This competitive protein binding assay is based on the following principles:

If a stable compound A is introduced into a system which contains a constant amount of radioactive compound A^* and is binding to protein P, A will displace A^* from the protein binding sites, in proportion to its concentration.

$P + A^* <-----> PA^*$ (labelled complex)

$P + A <-----> PA$ (unlabeled complex)

The amount of radioactivity bound in the labelled complex decreases as the amount of unlabeled compound is increased. Cyclic AMP in the sample and a fixed quantity of tritium labelled cAMP compete for binding to a protein with high specificity and affinity for cyclic AMP. The amount of labelled cAMP -

protein complex formed is inversely proportional to the amount of labelled cyclic AMP in the sample. The unbound nucleotide is removed by a precipitation reaction using activated charcoal. A supernatant, containing the bound nucleotide is obtained by centrifugation and removed for scintillation counting. A standard curve is constructed. Co/Cx is plotted against concentration of cyclic AMP in the standard dilutions. Co: labelled nucleotide bound in the absence of unlabeled nucleotide. Cx: labelled nucleotide bound in the presence of a standard quantity of unlabeled nucleotide.

Assay kits were obtained from the Radiochemical Center, Amersham, England and contain: Tris/EDTA buffer; purified bovine muscle protein; (8-^{3}H) cyclic AMP; cyclic AMP standard and charcoal absorbent.

iii) Endothelin.

Sample collection: Coronary effluent samples were collected in containers spiked with dipotassium ethylene diamine tetra-acetic acid (EDTA) and triton X100 (TTX) to give a final concentration of approximately 5 mmol/l and 0.5%, respectively. Samples were frozen at -70^{o}C until processed. The entire perfusate was loaded onto pre-conditioned (3 ml methanol, 5 ml water) Sep-Pak C_{18} cartridges (Waters Ass., Milford, MASS, USA), and endothelin was eluted with 2 ml 60% acetonitrile in 0.1% trifluoroacetic acid yielding a recovery of 57-67%. All endothelin-like immunoreactivity levels were corrected for a mean recovery of 60%. The eluates were freeze-dried and the dry residue stored at -20^{o}C pending analysis. Since the residue was redissolved in 0.5 ml of assay buffer (see below), the coronary effluent samples were concentrated 60 to 200 times. Columns were reused after extensive washing with methanol and water.

Endothelin determination: Endothelin (ie, endothelin-1, -2, and -3) of coronary effluents was measured using a commercial endothelin-2 (human) radioimmunoassay (RIA) kit (RIK 6910, Peninsula Laboratories, Belmont, CA, USA) and following established methods with slight modifications (Cernacek and Stewart, 1989; Kohno *et al.*, 1990). Samples were reconstituted in 0.5 ml phosphate buffer containing 19 mmol/l NaH_2PO_4, 81 mmol/l Na_2HPO_4, 50 mmol/l NaCl, 0.1% BSA, 0.01% NaN_3, and 0.1% TTX (pH 7.4). Duplicate samples were used in the assay. Standard curves were constructed using endothelin-2 to give a range of 0.25 to 16 pg/tube. Samples and standards (100 μl) were incubated with 100 μl assay buffer containing polyclonal anti-endothelin-2 antibody for 22 to 26 hours at 6°C before adding 100 μl of assay buffer containing ^{125}I-Endothelin-2 (specific activity approximately 1,000 Ci/mmol, 10,000 cpm/tube). The antiserum was diluted 3.5-fold, resulting in a fourfold increase in sensitivity compared to undiluted serum. The vortexing and incubation steps were repeated. Aliquots of buffer (100 μl) containing undiluted goat anti-rabbit antibody and normal rabbit serum, respectively were added to each tube and incubated at 6°C overnight. Upon addition of 500 μl assay buffer (0.2% BSA), bound and free radioactivity were separated by centrifugation at 3,500 rpm for 25 minutes. The supernatant was removed by aspiration and the pellet counted for ^{125}I in a gamma counter. To determine the amount of immunoreactive endothelin, the value of specific binding (total binding minus non-specific binding) was determined from the standard curve using a logistic fit. Total binding in the absence of competing endothelin amounted to 23.5±3.3% and non-specific binding to 3.2±0.5% (n=6). The IC_{50} was 1.9±0.28 pg/tube. The limit of detection was 0.81±0.13 pg/tube. Since all samples were highly concentrated, most concentrations to be measured were higher than the IC_{50}. The intra-assay coefficient of variation was determined by chromatography of 20

ml of perfusate spiked with 2 pg endothelin-2 and was 7.7% (n=3) and the inter-assay coefficient of variation in two consecutive runs was 13.0%.

Endothelin-1 (human, porcine; synthetic), endothelin-2 (human; synthetic) and endothelin-3 (human, rat; synthetic) were obtained from Sigma Chemical Company (St. Louis, Mo, USA). Foetal calf serum was from Highveld Biological Ltd, Kelvin (South Africa). The peptides were dissolved in perfusion medium with 0.1% BSA or RIA buffer as appropriate to prevent loss due to non-specific adhesion, aliquoted, freeze-dried and stored at -20^{o}C.

iv) Electrolyte composition and pH of arterial and coronary effluent samples.

Aortic and venous samples were taken at predetermined times, placed on ice and immediately analysed in an AMDEV Lytening 6 electrolyte analyser (Danvers, Massachusetts) and a Instrumentation Laboratory 1302 blood gas analyser (Milan, Italy). Electrolyte concentrations were presented in mmol/l.

C. Arrhythmias.

Ischaemic and reperfusion arrhythmia studies were performed using the Langendorff perfused rat heart model. Electrocardiograms were recorded throughout the ischaemic and reperfusion period to monitor the incidence of ventricular arrhythmias. Two electrodes were used; one attached to the aortic cannula and the other placed superficially into the right ventricle. The electrode was so placed to avoid damage to coronary vessels. Recordings were made on a Lectromed Multitrace 2 chart recorder.

Criteria for arrhythmias. Ventricular tachycardia was defined as 3 or more consecutive morphologically similar ventricular extrasystoles. These

extrasystoles were usually associated with a sharp rise in left ventricular diastolic pressure.

Ventricular fibrillation was defined as more than 6 consecutive ventricular complexes showing total irregularity of morphology. This pattern was associated with a loss of left ventricular pressure generation and mechanical failure (Lubbe, Daries and Opie, 1978) (Figure 4.3).

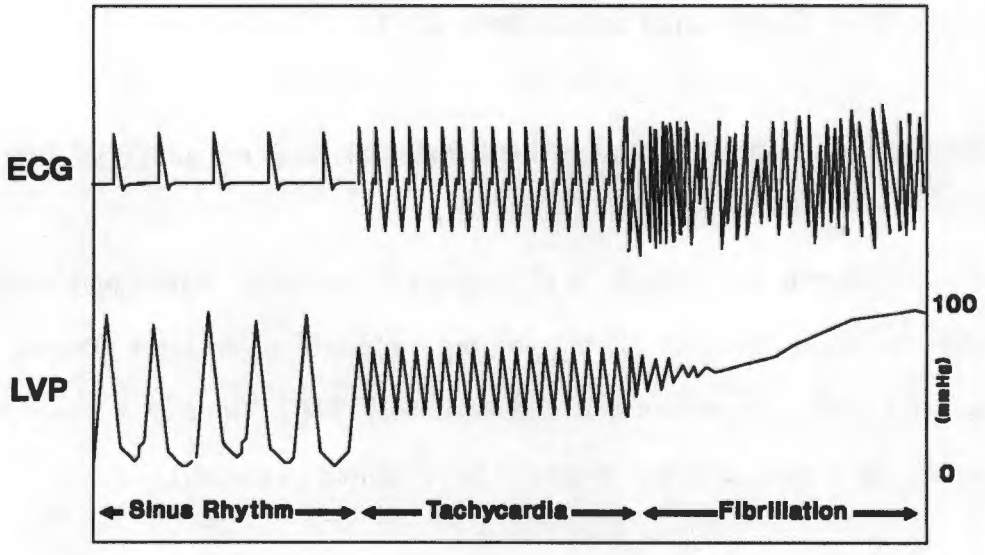

FIGURE 4.3 *Electrocardiogram (ECG) and left ventricular pressure (LVP) trace showing the reperfusion phase in a control heart. Pressure trace changes clearly show the occurrence of VT with a sharp increase in diastolic pressure while the transition to VF was marked by the complete loss of left ventricular developed pressure.*

In experiments where we were interested in reperfusion arrhythmias and mechanical function, fibrillating hearts were allowed 15 sec in ventricular fibrillation in which to spontaneously revert to normal sinus rhythm. Failing this,

hearts were defibrillated by submersion in ice cold ($\pm 4^{o}C$) Krebs-Henseleit buffer for 3-5 sec.

4.5 Characteristics of the Isolated Rat Heart Model.

A. Advantages of the model.

1) The model is not prone to hormonal and homeostatic changes that are operative in the intact animal and that may influence mechanical function and vulnerability of the heart to arrhythmias.

2) The model is not subjected to prolonged anaesthesia which may cause changes in blood pressure, heart rate, and mechanical function of the heart.

3) This model allows precise control of the perfusion solution composition and concentrations of drugs and substrates being used, and is less prone to fluctuations in perfusion conditions often experienced with more complicated in vivo models.

4) The rat is small, cheap to obtain and accommodate and freely available.

5) Haemodynamic parameters such as the preload and/or afterload of the perfusion system can be altered, and their effects on aortic output, coronary flow and ventricular pressure monitored.

6) The model is also suitable for investigating biochemical and metabolic profiles before ischaemia, during ischaemia, and during reperfusion.

B. Disadvantages of the model.

1) The crystalloid perfusate does not contain the essential amino acids and protein components present in blood and may therefore only be a suitable substitute for blood for a relatively short period. Hearts subject to prolonged periods of isolated crystalloid perfusion are prone to a deterioration in mechanical function.

2) The isolated heart is devoid of autonomic nerve control and data obtained under these conditions should be interpreted with caution.

3) The hearts perfused with a Krebs-Henseleit buffer, have unphysiologically high coronary flows due to the absence of haemoglobin and the low viscosity of the solution.

4) These hearts have no peripheral circulation and/or collateral flow.

4.6 Development of a model of stunning.

A. Mechanical studies: To establish a model of stunning, a series of experiments using different ischaemic times thought to induce reversible ischaemic damage were performed. Twenty minutes global ischaemia at $\pm 35^{o}C$ caused prolonged post-ischaemic dysfunction. Subsequent experiments were performed in which hearts were exposed to 20 minutes ischaemia at $\pm 35^{o}C$, reperfused, and aortic output, coronary flow and left ventricular developed pressure monitored over 2 hours. A concurrent control group was perfused for the same period without being exposed to ischaemia.

Mechanical evidence for stunning: Hearts exposed to 20 minutes ischaemia were perfused for two hours after reperfusion to determine whether the function would return to normal. There was a gradual increase in the cardiac output of the reperfused heart with a maximal value (± 85 % of the pre-ischaemic value) being reached after about an hour of reperfusion (Figure 4.4), after which the function again started to deteriorate. Perfusion of non-ischaemic working hearts also resulted in a gradual decline in the cardiac output. When the hearts were perfused for up to two hours, the function of the stunned and the control hearts appear to decline at approximately the same rate, in a parallel fashion (Table 4.1).

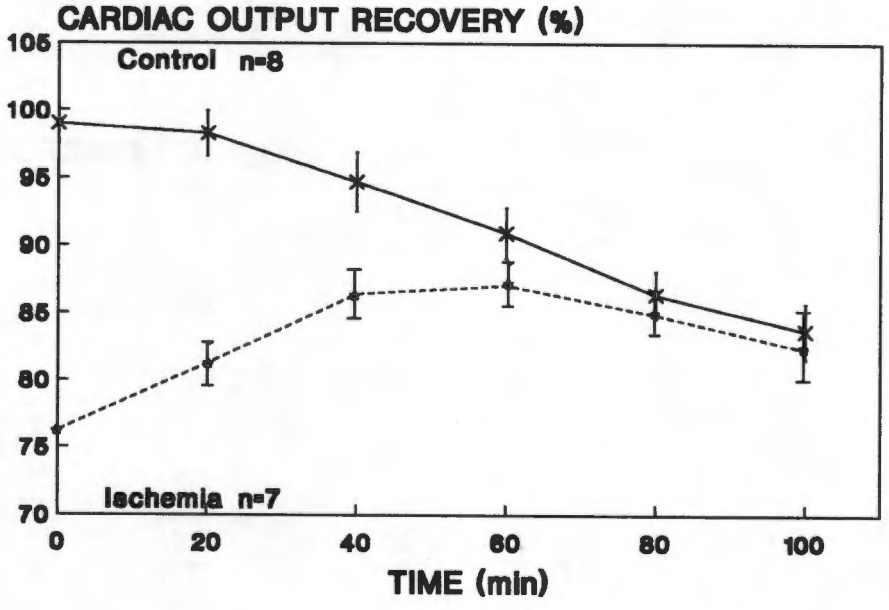

FIGURE 4.4 *Line graph represents two groups of hearts perfused for 120 minutes following ischaemia. Control hearts were perfused for 25 minutes in the working mode and then switched to Langendorff mode for 20 minutes, while the ischaemia group was subjected to 25 minutes perfusion followed by 20 minutes ischaemia. Hearts were now switched to work mode and mechanical function was monitored for 120 minutes.*

TABLE 4.1 Pre-ischaemic and reperfusion coronary flow (ml/min)(CF), aortic output (ml/min)(AO), cardiac output (ml/min)(CO), developed pressure (mmHg)(DP) aortic output recoveries (%)(AO REC) and cardiac output recoveries (%)(CO REC) of non-ischaemic control hearts(A) and time matched ischaemic hearts(B).

A. Control non-ischaemic hearts

Initial observations

	CF	AO	CO	DP	AO REC	CO REC
	15.6	45.1	60.7	82.4	-	-
	0.5	2.3	2.6	3.8	-	-
20min	15.6	44.0	59.6	80.4	97.7	98.3
	0.5	2.0	2.4	3.4	2.3	1.7
40min	15.5	41.7	57.2	79.0	92.9	94.7
	0.6	1.7	2.6	2.7	3.0	2.2
60min	15.6	39.5	55.1	76.7	87.8	90.9
	0.5	1.6	1.8	2.3	2.5	1.8
80min	15.6	36.9	52.5	74.3	81.6	86.4
	0.5	2.3	2.5	2.1	2.4	1.8
100min	15.1	35.6	50.7	74.3	78.6	83.6
	0.3	2.3	2.3	2.4	3.0	2.0

B. Model (20min of total global ischaemic)

Pre-ischaemic

	CF	AO	CO	DP	AO REC	CO REC
	16.2	47.1	63.3	79.1	-	-
	0.9	2.2	2.7	5.0	-	-
Reperfusion						
20min	16.2	36.1 *	52.3 *	74.4	74.1 **	82.7 **
	0.9	2.0	2.1	5.1	1.4	2.3
40min	16.5	39.7	56.2	76.0	82.4 *	88.8
	0.5	1.8	2.6	4.3	1.6	2.0
60min	16.6	40.0	56.6	76.3	83.2	89.0
	1.2	2.7	3.4	4.3	3.0	2.4
80min	16.6	39.1	55.7	75.0	82.9	87.5
	1.2	2.8	3.5	4.5	2.9	2.1
100min	17.0	37.1	54.1	77.7	76.1	83.2
	0.3	4.1	5.1	5.4	5.6	4.4

Values are means $\pm$ SEM for n=7 for panel A. and n=8 for panel B.
Note: Cardiac output recoveries reach control values after 60 minutes, and aortic outputs recoveries reach control values after 80 minutes.
* $p < 0.05$ vs control
** $p < 0.002$ vs control

B. Morphological studies: One of the important provisos in the definition of reperfusion stunning is that the reperfused myocardium must be entirely viable (Bolli, 1990). Bolli (1990) goes as far as to say that stunning is relatively mild, sublethal injury that must be kept quite distinct from myocardial infarction. In order to determine whether the reperfused hearts we were dealing with were indeed stunned and not infarcted, we did a series of experiments in which we performing ultrastructural studies. The objective was to determine whether the ultrastructural changes observed at the end of ischaemia and during reperfusion were similar to those defined by Schaper and co-workers (1979) and Jennings and co-workers (1985). These ultrastructural changes which are characteristic of reversibly injured myocytes are discussed in chapter 2 of this thesis.

At predetermined stages (immediately before ischaemia, at the end of ischaemia, and after 20 minutes reperfusion) of the experimental protocol, hearts were fixed for transmission electron microscopy by perfusion with 1% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.4) for 5 minutes. After removal from the cannula, the heart was sectioned transmurally, and $1mm^3$ samples were fixed in osmium tetroxide, dehydrated, and embedded in ERL resin (Harper, Williams and Lochner, 1990).

Ultrastuctural evidence of stunning: After perfusion fixation, electron microscopy showed that the myocardium had developed intracellular oedema, swollen mitochondria, and loss of mitochondrial granules during ischaemia. There was no obvious myofibrillar damage. After 20 minutes of reperfusion, oedema was absent, and the myofibrils were relaxed with no signs of mitochondrial abnormalities. There were extremely few necrotic myocytes, not more than 1% of the total. (Figures 4.5 and 4.6)

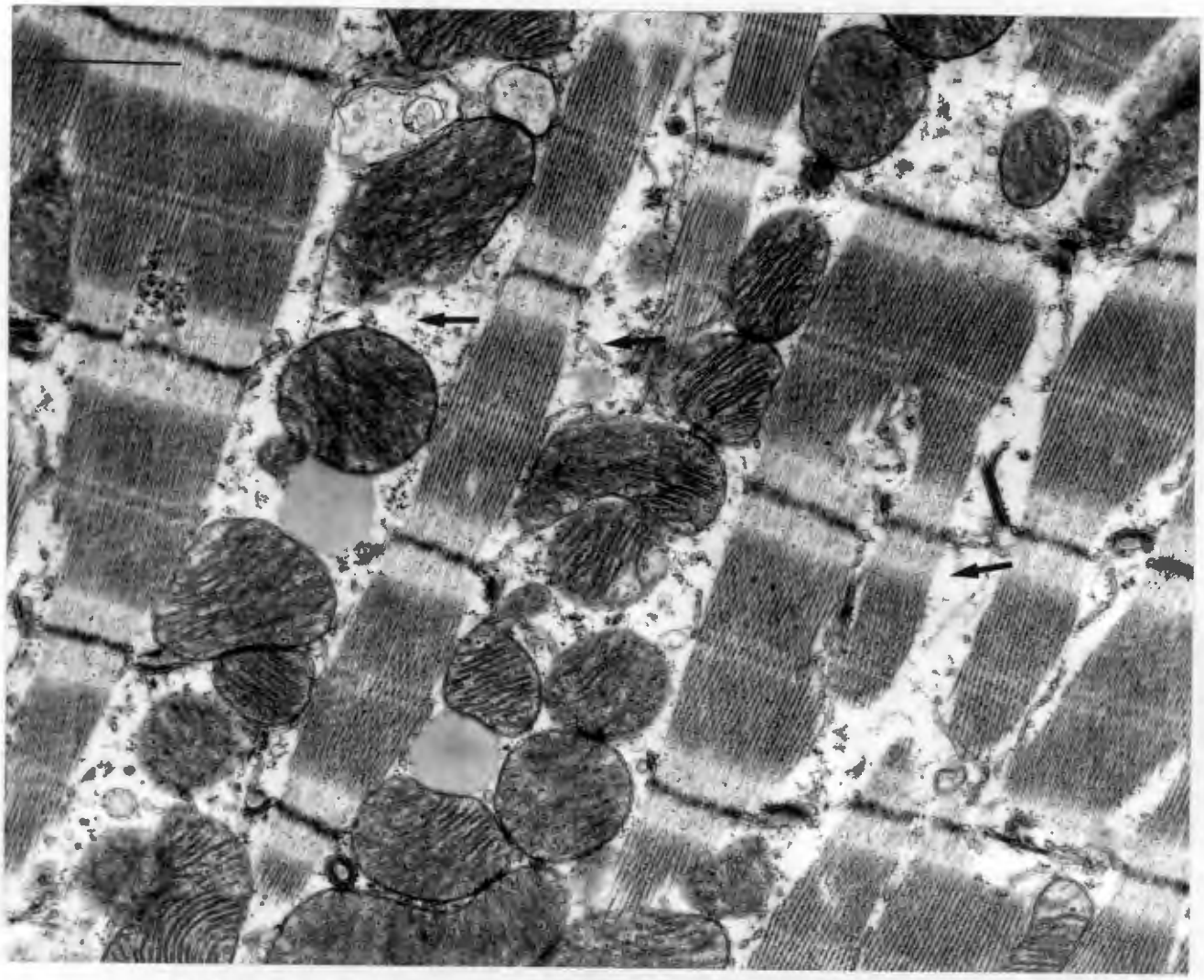

FIGURE 4.5 *Ultrastructural detail from a subendocardial region showing the effects of 20 minutes of global ischaemia. Note the slight intracellular oedema (arrows), swollen mitochondria and loss of mitochondrial granules. No obvious myofibrillar damage is visible.*
Bar = 1μm.

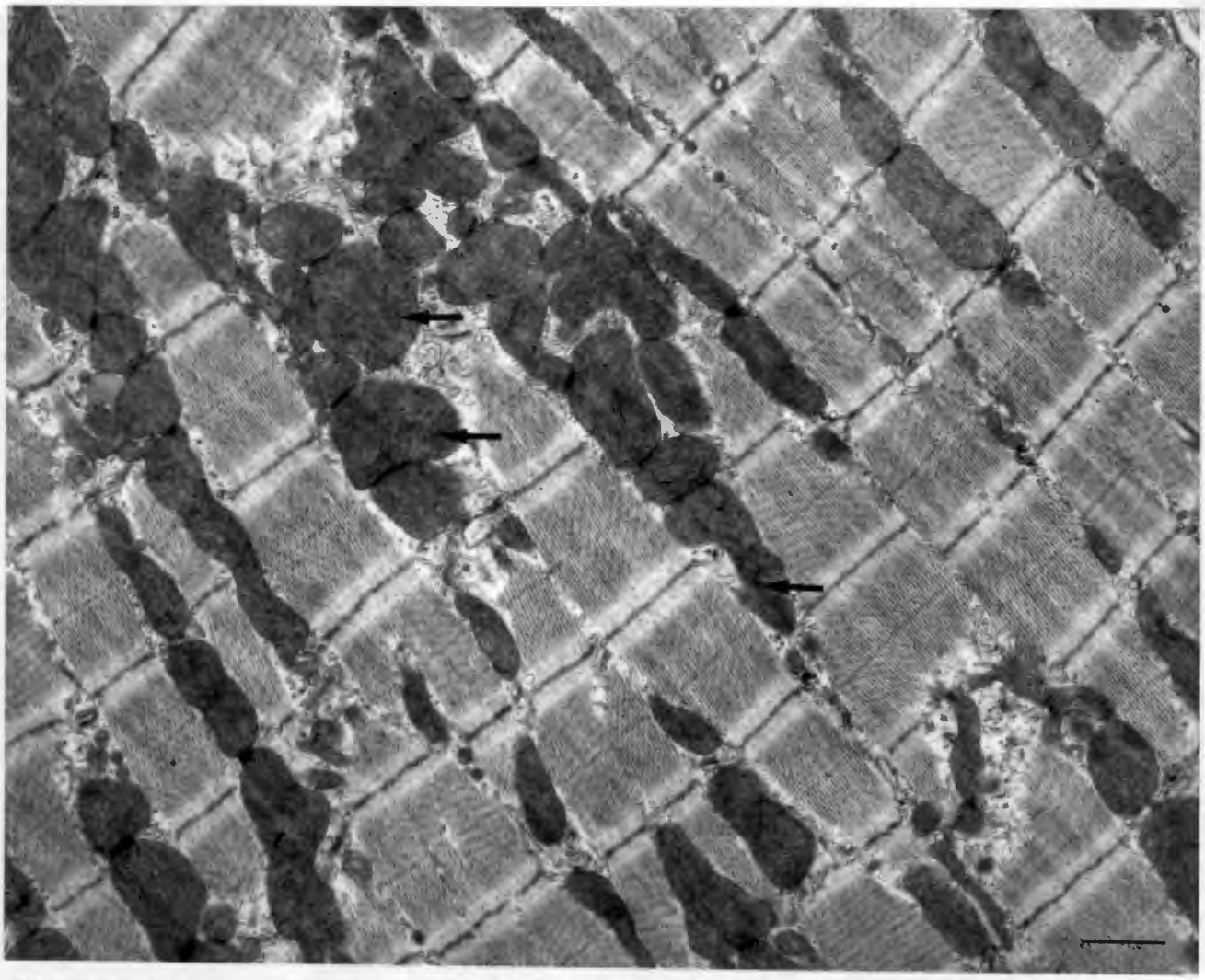

FIGURE 4.6 *Normal ultrastructure from the subendocardial region of a heart reperfused for 20 minutes following global ischaemia. Note the relaxed myofibrils and the absence of oedema. Mitochondria have a compact appearance and contain normal dense granules (arrowed).*
Bar = 1μm.

C. Response of the model to inotropic interventions: To determine whether the already stunned hearts in our model were able to respond to inotropic interventions, we performed 3 groups of experiments in which we subjected already stunned hearts to isoproterenol (10^{-7}M)(a ß-receptor stimulant, Winthrop), Bay K 8644 (10^{-8}M) (a Ca^{2+} agonist, Bayer), or high extracellular Ca^{2+} concentrations (2.25mM) 15 minutes after reperfusion.

Inotropic response of the stunned heart: Isoproterenol 15 minutes after reperfusion increased AO from 15.9±2.1 to 66.7±2.3 ml/min (n=8), Bay K 8644 increased AO from 33.6±2.1 to 52.0±2.8 ml/min (n=5), and an increase in extracellular Ca^{2+} (2.25 mM) increased CO from 26.3±2.1 to 39.4±1.8 ml/min (n=7)(Figure 4.7).

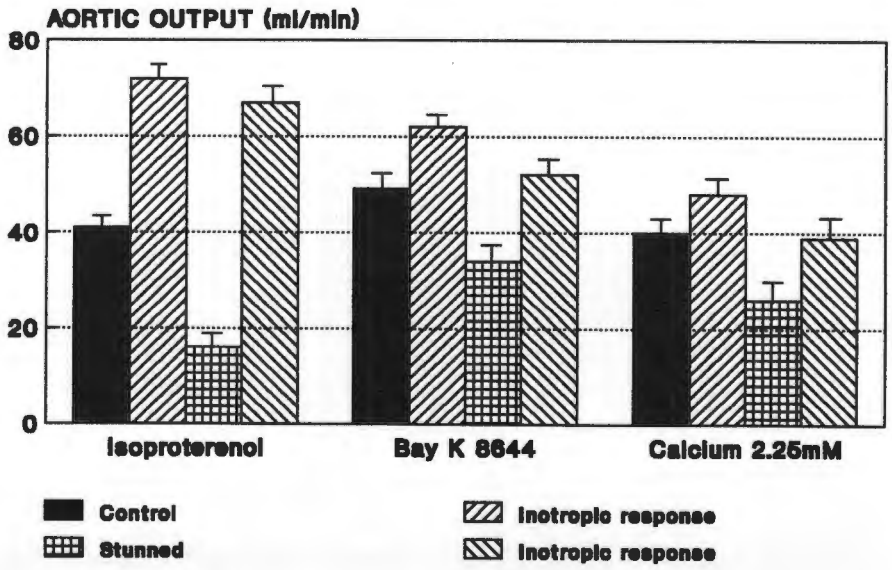

FIGURE 4.7 *Histogram presenting aortic outputs (AO) of hearts under control conditions (filled column), during pre-ischaemic inotropic stimulation (cross-hatched column), stunned after ischaemia (grid-patterned column), and stunned and inotropically stimulated (cross-hatched column). Hearts were treated with isoproterenol, Bay K 8644 or 2.25mM* Ca^{2+}.

When isoproterenol, Bay K 8644, or forskolin were used in the late reperfusion of the already stunned heart, the expected positive inotropic response was obtained without significant negative effects on the post-treatment mechanical function. Aortic output values similar to the values obtained in the pre-ischaemic (untreated) hearts were achieved during inotropic treatment of these hearts.

These data are supported by the work done by Mercier and co-workers (1982), Taegtmeyer and co-workers (1985), and Becker and co-workers (1986) who showed that the already stunned heart still had the ability to respond to inotropic interventions.

Reservations and benefits of the model of stunning used in these studies:
Although the in vivo coronary occluded dog heart model is considered the classic model of myocardial stunning (Heyndrickx et al., 1975), the simpler isolated ferret (Kusuoka et al., 1987; 1990), rabbit (Ambrosio et al., 1987) and rat heart (Nayler, Buckley and Leong, 1990; Steenbergen, Fralix and Murphy, 1993) model has been used. Despite controversy regarding the suitability of the isolated heart as a model of stunning, isolated hearts reperfused after transient ischaemia exhibit complete normalisation of phosphocreatine content and pH (Taegtmeyer et al., 1985; Porterfield et al., 1987; Ambrosio et al., 1987), indicating that viability is generally preserved. Despite the fact that reversibility of contractile abnormalities cannot be verified, the metabolic recovery of these hearts suggests that the injury is mostly nonlethal (Bolli, 1990). Bolli (1990) concludes that despite numerous obvious differences from ischaemia in vivo, isolated heart preparations can mimic myocardial stunning under selected circumstances.

The failure of the working heart to maintain normal mechanical function after some hours may be due to the composition of the perfusion solution which differs in many ways from blood, for example by not containing amino acids. Furthermore, the abnormally high coronary flow of the crystalloid perfused heart may by the hydraulic or erectile effect (Vogel *et al.*, 1982) place an excess workload on the myocardium. However, an advantage of the crystalloid perfusion preparation is the absence of neutrophils, which may play a role in certain models of reperfusion injury. In addition, the isolated heart model

eliminates the possibility that peripheral effects of certain drugs may complicate the interpretation of the results.

4.7 Statistical Methods.

The results are presented as a mean ± standard error of the mean (SEM). Significances between groups was determined using the unpaired Student's t-test. When multiple comparisons were made, the Bonferroni correction or ANOVA test were made.

The Fisher's exact test was used to assess the significance of the differences in the incidence of ventricular arrhythmias between groups (Wallenstein, Zucker, Fleiss, 1980).

CHAPTER 5-9

EXPERIMENTAL DATA

CHAPTER 5.

Reperfusion Stunning: Its Modulation by Agents Altering Ca^{2+} Flux at the Onset of Reperfusion

a) Rationale.

This study tested the hypothesis that a reduction in Ca^{2+} flux across the sarcolemma and/or the sarcoplasmic reticulum at the onset of reperfusion could attenuate subsequent mechanical stunning.

Although stunning may be lessened by pretreatment with Ca^{2+} antagonists (Nayler, Buckley and Leong, 1990), these results may reflect amelioration of the severity of ischaemic injury and hence, less reperfusion injury (Opie, 1989b; Manning and Hearse, 1984). A specific role for Ca^{2+} overload is suggested by three observations. First, transient Ca^{2+} overload induced by perfusing the isolated heart with a high Ca^{2+} perfusate results in contractile dysfunction and ATP depletion (Kitikaze, Weisman and Marban, 1988). Second, during ventricular fibrillation there is an increase in intracellular Ca^{2+} concentration with subsequent contractile dysfunction in the post-fibrillating period (Koretsune and Marban, 1989). Third, measurement of cytosolic Ca^{2+} in the stunned rat heart show high Ca^{2+} transients (Kusuoka *et al.*, 1990) Nonetheless, data are conflicting in that Ito and co-workers (1987) found that an increased external Ca^{2+} improved the mechanical function of the stunned dog myocardium. Catecholamine stimulation, a procedure which should increase cytosolic Ca^{2+} levels, is also able to overcome the mechanical impairment in the canine heart (Mercier *et al.*, 1982; Ellis *et al.*, 1983b).

The major hypothesis tested in this study was that transient Ca^{2+} overload at the onset of reperfusion could give rise to stunning. An additional hypothesis was that once stunned, the myocardium behaved as if there were a deficiency of Ca^{2+} available to the contractile apparatus, so that at this stage the mechanical function could be improved by agents thought to enhance cytosolic Ca^{2+}. To determine whether Ca^{2+} fluxes at the onset of reperfusion could be modulated to exaggerate or attenuate stunning, we subjected isolated hearts in the first 2 minutes of reperfusion to procedures thought to increase Ca^{2+} influx (high extracellular Ca^{2+}, isoproterenol, forskolin, or the Ca^{2+} agonist Bay K 8644) or to inhibit Ca^{2+} flux, (low external Ca^{2+}, nisoldipine or ryanodine, or inorganic blockers Mn^{2+} or Mg^{2+}). We also subjected hearts already stunned to some of these procedures 15 minutes after reperfusion.

b) Experimental procedures, materials and methods.

i) Model Used: The model and the perfusion solutions and protocol are as described in chapter 4.

Myocardial temperature during ischaemia: In this study, hearts were subjected to 20 minutes of total global ischaemia at 35 ± 0.5 ^{o}C before being reperfused at $37^{o}C$ for 5 minutes in the Langendorff mode. During total global ischaemia the myocardial temperature decreased to $30^{o}C$ unless the temperature of the water-jacket surrounding the heart was increased to 40-$42^{o}C$ (depending on the ambient temperature). During the first 2 minutes of reperfusion, the different pharmacologic compounds were infused (again at $37^{o}C$) or the ionic composition of the reperfusion solution was altered with the aim of modulation of Ca^{2+} flux across the sarcolemma or sarcoplasmic reticulum. Thereafter, the standard perfusion medium was used again, free of any such changes (For a

diagrammatic representation of the experimental protocol, see Figure 4.2 of chapter 4).

ii) Interventions in the initial 2 minutes of reperfusion: Each of the following series of experiments had its own group of concurrent controls.

a) Varying (1.25-10.0 mM) Ca^{2+} concentrations in a Tyrode's reperfusion solution.

b) Reperfusion with isoproterenol (10^{-7} M), forskolin (10^{-8} M) or Bay K 8644 (10^{-8} M) was investigated.

c) Hearts were reperfused with low Ca^{2+} (0.75 mM), nisoldipine (10^{-8} M) (Bayer, Germany) or Ca^{2+} channel blocking cations (magnesium 16 mM or manganese 2 mM), or ryanodine $3x10^{-9}$ M (Calbiochem).

d) Hearts were reperfused with a high K^{+} concentration (21 mM) solution to maintain arrest because asystole itself (as seen with Mn^{2+} and Mg^{2+}) might be protective. The effect of each of the two Ca^{2+} blocking cations added to this high K^{+} containing solution was also investigated. The effects of a low K^{+} (2mM) concentration in the reperfusion solution was also tested.

iii) Additional interventions:

a) Hearts were pre-treated with nisoldipine or with ryanodine given over 2 minutes before the onset of ischaemia.

b) Post-ischaemic stunned hearts were treated with nisoldipine (10^{-8} M) 15 minutes after reperfusion for 2 minutes and cardiac output was measured at the end of the 2 minutes and then again after an 8 minute washout period.

c) Results

Haemodynamic data: There was a linear relationship between cardiac output and developed pressures. There were no significant changes in coronary flow between the various groups of hearts. Changes in heart rate during mechanical function measurements were avoided by electrical pacing (Figure 5.1).

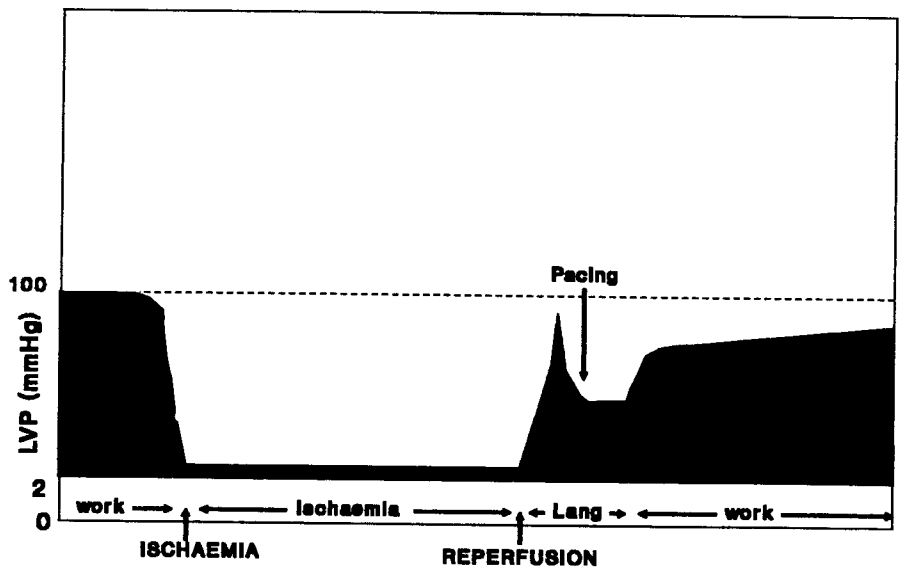

FIGURE 5.1 Diagrammatic representation of a typical left ventricular pressure tracing recorded during an experiment. On the left is the pre-ischaemic systolic and diastolic left ventricular pressure tracing (measured in millimeters of mercury), followed by ischaemia and reperfusion (in the Langendorff [Lang] mode) and then post-ischaemic work.

i) Changes in early reperfusion Ca^{2+} concentration: With increases (above 1.25 mM) in the extracellular Ca^{2+} concentrations of the ***early*** reperfusion solution there was a dose dependent decrease in post ischaemic functional recovery (Figure 5.2). CO recovery was 82.3±1.8% (n=8) with a Ca^{2+} concentration of 1.25 mM, 76.1±2.8% (n=9) with a Ca^{2+} of 3.75 mM with a further decrease to 56.3±3.7% ($p< 0.05$) (n=7) with a Ca^{2+} concentration of 10.0 mM.

Haemodynamic parameters (aortic output, coronary flow and developed pressure) are presented in Table 5.1.

ii) Inotropic intervention in early reperfusion: Figure 5.2 shows that isoproterenol in the reperfusion solution gave CO recoveries of 53.4±3.6% ($p<0.05$) (n=8) which were similar to those reperfused with a Ca^{2+} concentration of 10.0 mM. Forskolin decreased CO recoveries from 80.9±0.9% (control) (n=7) to 62.7±2.4% ($p<0.002$) (n=8). Bay K 8644 added in the reperfusion period decreased CO recovery from 66.72±2.4% (n=4)(control) to 43.4±5.9% ($p<0.05$) (n=6).

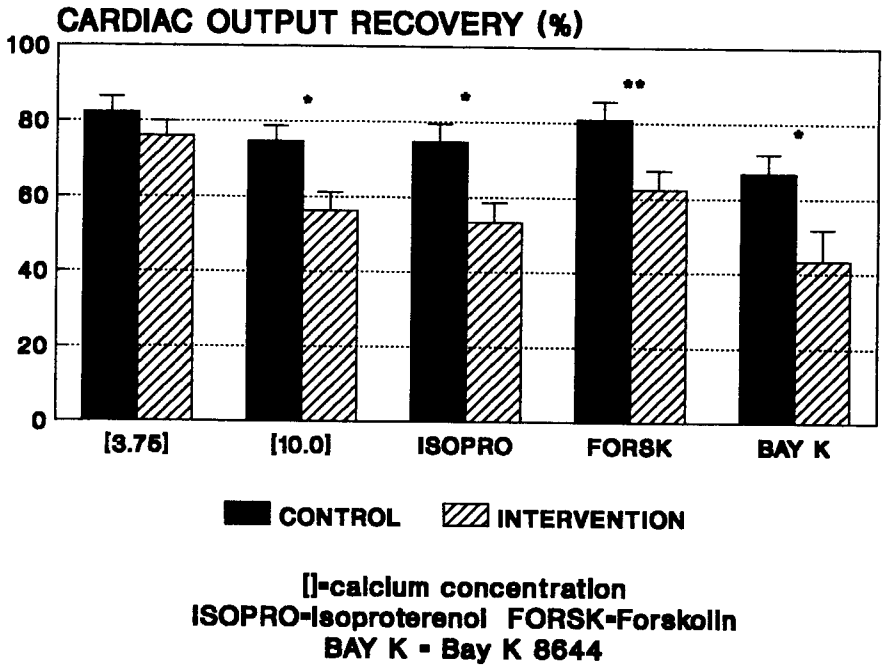

FIGURE 5.2 *Cardiac output expressed as % recovery of pre-ischaemia values for hearts initially reperfused with Tyrode's solution containing varying Ca^{2+} concentrations or Krebs-Henseleit with one of the inotropes (cross-hatched columns). Mechanical function measurements were done 10 minutes into the pre-ischaemia work period and again 10 minutes into the post-ischaemia work period. For each group of experiments performed, there was a concurrent control group of hearts [Ca^{2+}-1.25mM] (filled columns).*

** $p<0.05$ treated vs control.*

*** $p<0.002$ treated vs control.*

iii) Ca^{2+} antagonists in early reperfusion: The use of a lower (0.75 mM) extracellular Ca^{2+} concentration in the reperfusion solution resulted in improved CO recoveries of 91.8±0.8% ($p< 0.05$) (n=6) (control 79.2±2.6%), while the reperfusion with nisoldipine gave a CO recovery of 99.0±2.8% ($p< 0.002$) (n=8) (control 82.11±2.0%) (Figure 5.3). Manganese or magnesium added to the reperfusion solution improved CO to 88.9±2.4% ($p< 0.05$) (n=8) and 91.9±1.4%

($p< 0.002$) ($n=6$) respectively in comparison with the control value of $75.4\pm2.6\%$ ($n=10$). Ryanodine in the reperfusion solution resulted in CO recoveries of $103.6\pm1.8\%$ ($p<0.002$) ($n=15$). Haemodynamic parameters for hearts reperfused with these Ca^{2+} flux modulators are presented in Table 5.2.

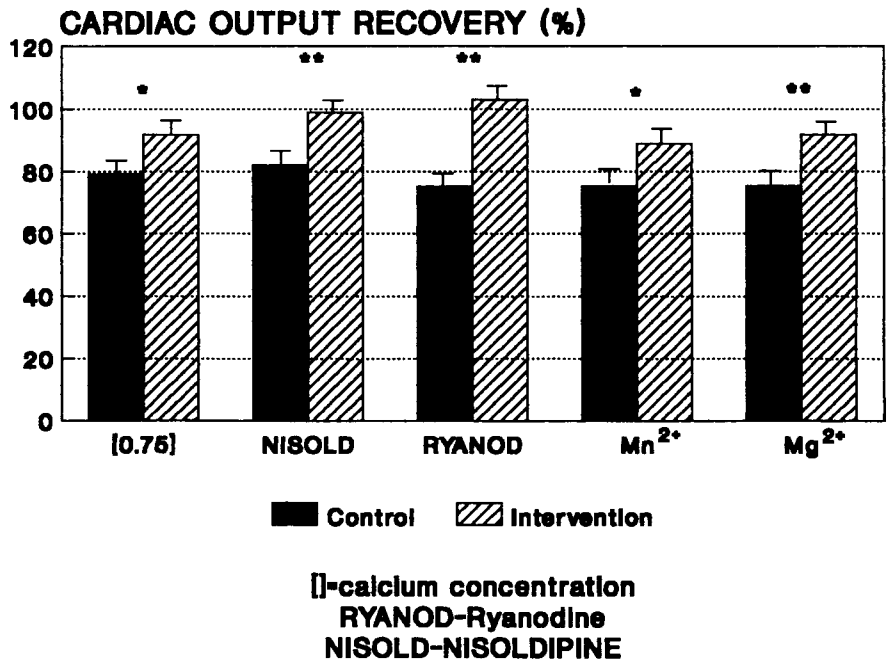

FIGURE 5.3 *Cardiac output recoveries expressed as a % of the pre-ischaemia values for hearts reperfused with low Ca^{2+}, a Ca^{2+} antagonist, a SR Ca^{2+} flux modulator or Ca^{2+} channel blocking cation (cross-hatched columns) and their controls (filled columns). Function measurements were done 10 minutes into the pre-ischaemia work period and again 10 minutes into the post-ischaemia work period.*

** $p<0.05$ treated vs control.*

*** $p<0.002$ treated vs control.*

TABLE 5.1 Post-ischaemic aortic outputs, coronary flows and developed pressures for different reperfusion solutions.

Reperfusion solution	Aortic output(ml/min)	Coronary flow(ml/min)	Developed pressure(mmHg)
Ca^{2+} [3.75mM] n=9	34.9±2.7	17.8±1.2	83.2±2.9
Control n=6	29.8±2.3	17.2±0.6	77.2±1.2
Isoproterenol n=8	14.5±2.3	18.2±0.9	60.4±0.9
Ca^{2+} [10mM] n=7	18.0±2.9	15.7±0.7	59.3±3.1
Control n=6	29.8±2.3	17.1±0.6	67.2±1.6
Forskolin n=8	21.4±1.7	16.2±0.4	71.3±1.2
Bay K 8644 n=6	16.1±3.7	17.1±0.6	70.3±1.5
Control n=7	34.3±1.4	16.9±0.6	75.0±1.2

Table 5.1 represents hearts (with controls) reperfused with Ca^{2+} agonists or inotropes in the early reperfusion solution. All control groups were reperfused with a reperfusion solution containing a Ca^{2+} concentration of 1.25mM.

TABLE 5.2 Post-ischaemic aortic outputs, coronary flows and developed pressures for different reperfusion solutions.

Reperfusion solution	Aortic output(ml/min)	Coronary flow(ml/min)	Developed pressure(mmHg)
Ryanodine n=15	43.6±1.9	17.9±0.8	94.3±2.0
Control n=6	37.3±2.8	18.3±1.7	92.0±4.1
Nisoldipine n=8	46.0±2.8	17.2±0.9	97.4±2.0
Control n=8	34.4±3.9	17.2±1.1	93.0±0.1
Low Ca^{2+} n=6	43.5±2.4	20.7±1.3	86.0±1.6
Control n=8	29.4±2.1	15.5±0.5	74.5±1.7
Mn^{2+} n=8	37.4±1.4	15.5±0.6	82.1±1.8
Mg^{2+} n=6	38.7±1.8	18.7±0.3	79.3±1.0
Control n=10	26.8±1.4	16.0±0.3	69.9±2.5

Table 5.2 represents hearts (with controls) reperfused with Ca^{2+} antagonists in the early reperfusion solution. All control groups were reperfused with a reperfusion solution containing a Ca^{2+} concentration of 1.25mM.

iv) Effect of a low or a high potassium concentration in the reperfusion solution together with Ca^{2+} channel blocking cations: (Figure 5.4) A low potassium concentration in the reperfusion solution decreased reperfusion CO recovery from 74.7±3.4% (n=6) to 60.49±1.1% ($p< 0.05$) (n=10). A high potassium concentration (21 mM) in the reperfusion solution gave CO recoveries of 62.6±6.9% (n=7) compared to the control values of 74.7±3.4% (n=6). Addition of Mg^{2+} or Mn^{2+} to a high potassium containing reperfusion solution improved post-ischaemic CO recoveries from a control value of 75.8±2.8% to 91.0±2.0% ($p< 0.002$) (n=6, $Mg^{2+}+K^{+}$ group) and 92.3±1.3% ($p< 0.002$) (n=11, $Mn^{2+}+K^{+}$group).

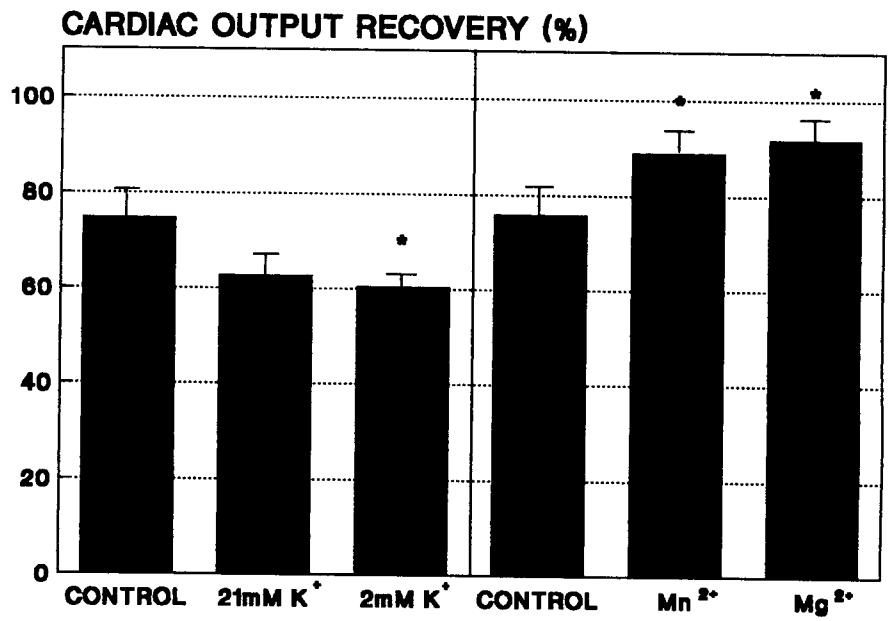

FIGURE 5.4 *Cardiac output recoveries expressed as a % of pre-ischaemia values for hearts reperfused using a low or high potassium concentration or high potassium in conjunction with one of the Ca^{2+} channel blocking cations (function was measured 10 minutes into the work period before and after ischaemia).*

** $p<0.002$ treated vs control.*

vi) Additional interventions.

a) The effect of internal or external Ca^{2+} blocker pre-treatment: Ryanodine gave CO recoveries of 97.5±1.8% ($p<0.002$) (n=11) and nisoldipine 84.8±5.1% (n=6) compared to control values of 82.3±1.8% (n=8) and 82.1±2.0% (n=8).

b) Ca^{2+} antagonist added to the already stunned heart: (Figure 5.5) Nisoldipine in late reperfusion (after 15 minutes) decreased cardiac output from 47.7±1.8 to 39.3±2.4 ml/min (n=6). This decrease in cardiac output was reversed on removal of nisoldipine from the perfusate.

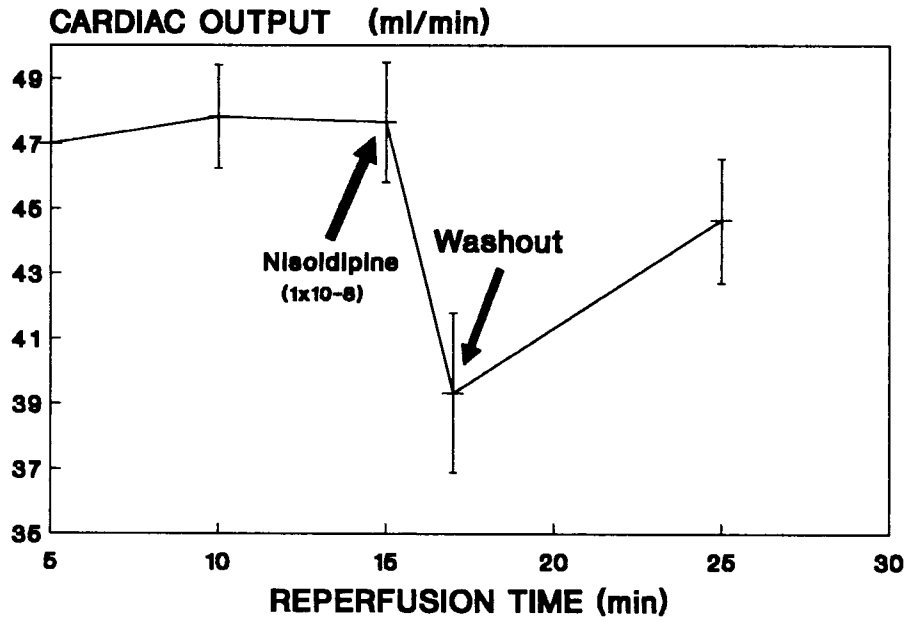

FIGURE 5.5 Decreased cardiac output with late Ca^{2+} antagonist treatment (with nisoldipine) of the isolated rat heart. Cardiac function reverts to normal on washout (removal from perfusate) of the antagonist.

Summary of findings of study. 1) Increased cytosolic Ca^{2+} overload at the onset of reperfusion by compounds that increase trans-sarcolemmal Ca^{2+} fluxes, exacerbates reperfusion stunning.

2) Compounds that decrease reperfusion trans-sarcolemmal Ca^{2+} influx or release from the SR decreases the severity of stunning.

CHAPTER 6

The Na^+/H^+ Exchanger and its Role in Reperfusion Stunning and Arrhythmias.

a) Rationale.

It is proposed that a rise in cytosolic Ca^{2+} during ischaemia and especially during reperfusion contributes toward reperfusion injury (Nayler, Poole-Wilson and Williams, 1979; Poole-Wilson *et al.*, 1984; Jennings, Reimer and Steenbergen, 1985; Kusuoka *et al.*, 1987; Steenbergen *et al.*, 1987; Kitikaze, Weisman and Marban, 1988; Opie and Coetzee, 1988; Marban *et al.*, 1989; Marban, 1991a; 1991b; Opie, 1991c; Chapter 3). There is good evidence that the increase in total tissue Ca^{2+} content during reperfusion is closely correlated to functional and morphological damage (Nayler, 1983; Tani, 1990; Steenbergen *et al.*, 1990). Alternatively, a transient rise in cytosolic Ca^{2+} during reperfusion may cause reversible injury in the form of depressed mechanical function and arrhythmias. This concept is supported by the data showing that interventions that limit trans-sarcolemmal Ca^{2+} ion fluxes during reperfusion, also improve mechanical function (Chapter 5) and lessen arrhythmias (Opie and Coetzee, 1988).

Hypothetically, Ca^{2+} may enter reperfused cells via Ca^{2+} channels (Chapter 5), or through trans-sarcolemmal passive influx along the concentration gradient, or indirectly via activity of the Na^+/H^+ exchanger and consequently that of the Na^+/Ca^{2+} exchanger (Tani and Neely, 1989; Dennis *et al.*, 1990; Murphy *et al.*, 1991; Karmazyn, 1988). Recent evidence also suggests that Na^+ may enter the cell via the pathologically modified Na^+-channels during ischaemia, activate Na^+/Ca^{2+} exchange during reperfusion, and thus also contribute to Ca^{2+} overload (Ver Donck and Borgers, 1991).

Pretreatment by amiloride decreased reperfusion mechanical stunning in the isolated Langendorff perfused rat heart (Tani and Neely, 1989; Murphy *et al.*, 1991; Scholz *et al.*, 1992). Amiloride derivatives more specifically inhibiting Na^+/H^+ exchange, decreased ischaemic (Karmazyn, 1988) and reperfusion arrhythmias (Dennis *et al.*, 1990). There is therefore indirect evidence for the role of Na^+/H^+ exchange in reperfusion injury.

The aim of the present study was to compare the effects of potent and very specific Na^+/H^+ exchange inhibitor, HOE 694 (Schmid *et al.*, 1992) with those of amiloride, an established inhibitor of this exchanger in the heart (Tani and Neely, 1989; Dennis *et al.*, 1990). The end points of our study were reperfusion stunning and arrhythmias in the isolated rat heart model. We also wished to ascertain whether the protective effect afforded by amiloride was purely due to its Na^+/H^+ exchange inhibitory properties or to its various nonspecific properties. Because amiloride decreases the rise in cytosolic Ca^{2+} concentrations during ischaemia (Murphy *et al.*, 1991), it is possible that some of the effects previously found could be due to limitation of ischaemic damage thereby lessening reperfusion injury (Opie, 1989b). We therefore also tested both reperfusion and pretreatment by amiloride or HOE 694 for effects on reperfusion stunning and arrhythmias.

b) Experimental procedures, materials and methods.

i) Models used.

Isolated perfused (working, or Langendorff-mode aortic perfused) rat hearts were subject to global ischaemia and reperfusion (Neely *et al.*, 1973).

During experiments using the Langendorff model, left ventricular developed pressure was used as an index of mechanical function. For these experiments an ultra-thin "Clingfilm" balloon was inserted into the left ventricle through the mitral valve and inflated to a diastolic pressure of 4-8 mmHg (Qiu and Hearse, 1992).

ii) Experimental protocols

1) Experimental protocol used for the working heart model: The protocol was as described in chapter 4 with the exception that hearts were exposed to 20 minutes of total global ischaemia at an epicardial left ventricular temperature of $36.5 \pm 0.5^{o}C$.

During the last 5 minutes of perfusion before ischaemia (pre-treatment), or for the first 2 minutes of the 5 minutes Langendorff reperfusion, one of the Na^{+}/H^{+} exchange inhibitors or the vehicle used to dissolve the compound (placebo) was infused into the heart. When hearts were reperfused with inhibitors, the standard perfusion medium was used for the remainder of the experiment after the initial 2 minute drug infusion. Reperfusion mechanical function was assessed in the work mode for 30 minutes.

2) Experimental protocol used for Langendorff model: Hearts were perfused in the Langendorff mode for 20 minutes (while left ventricular pressures were recorded). Hearts were treated with amiloride for 5 minutes before being made ischaemic for a further 30 minutes. The 30 minute ischaemic period was selected to match the ischaemic time used by Tani and Neely (1989) in their experiments using the same model and concentrations of amiloride during pretreatment. Hearts in this series were now reperfused for 30 minutes and mechanical function (left ventricular pressure) monitored. These hearts were not paced.

Developed pressure and heart rate were used to calculate the stroke-work index (SWI) before and after ischaemia. The SWI recovery was expressed as a percentage of the pre-ischaemic SWI value.

SWI = Developed pressure X Heart rate.

$$\% \text{ RECOVERY of SWI} = \frac{\text{(Reperfusion SWI)X100}}{\text{(Pre-ischaemic SWI)}}$$

iii) Coronary effluent collection: Effluent samples were collected from 0-15 sec, from 15-30 sec, from 30-60 sec and from 60-120 sec after the initiation of reperfusion. These and an aortic sample were immediately analysed on an IL 1302 blood-gas analyser (Instrumentation Laboratory, Milan, Italy.) to determine pO_2, pCO_2, and pH and an AMDEV Lytening 6 electrolyte analyser (Danvers, Massachusetts, USA) to measure coronary effluent Na^+, K^+ and Ca^{2+}.

iv) Reperfusion arrhythmias: During the first 2 minutes of reperfusion, a left ventricular pressure trace was recorded (chart speed of 10mm/sec) to monitor the incidence of ventricular tachycardia (VT) and/or ventricular fibrillation (VF) (For definitions, see chapter 2).

v) Materials: Amiloride was obtained from Sigma Chemicals (St Louis, Missouri), while the HOE 694 was supplied by Hoechst AG (Frankfurt, Germany). Amiloride was dissolved in dimethyl sulfoxide (DMSO) (highest concentration used was 0,1 % V/V) and the HOE 694 was soluble in deionised water. Both compounds altered perfusate pH which required correction of the stock solution when fresh solutions were made up every day.

c) Results

1) Effect of HOE 694 and amiloride pretreatment on reperfusion function and arrhythmias in hearts with left atrial cannulation.

HOE 694. After 30 minutes reperfusion, AO recoveries were 36.0±3.8% (5) for control hearts and 56.5±2.6% (5) ($p<0.05$) for hearts treated with 10^{-8}M HOE, 38.5±3.6% (5) for control hearts and 50.6±3.9% (7) ($p<0.05$) for hearts treated with 10^{-7}M HOE, and 38.2±2.4% (5) for control and 52.9±2.7% (5) ($p<0.05$) for hearts treated with 10^{-6}M HOE (Figure 6.1). There was thus no dose dependent effect.

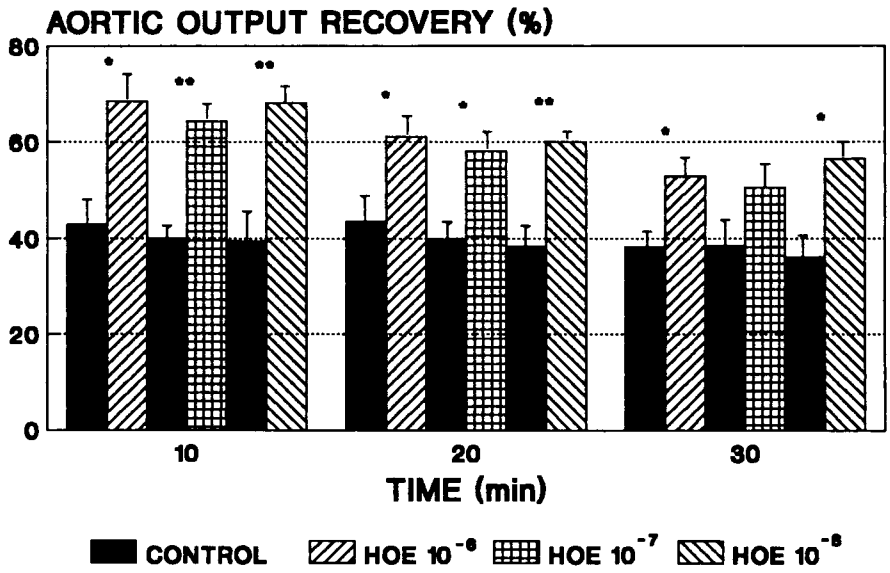

FIGURE 6.1 *Histogram showing aortic output (AO) recoveries (as a percentage of their pre-ischaemic value) after 10, 20 and 30 minutes reperfusion in hearts pretreated with HOE 694 (10^{-6} to 10^{-8}M) and their control groups.*
HOE=HOE 694.
** $p<0.05$ vs control.*
*** $p<0.02$ vs control.*

Pretreatment by HOE 10^{-8}M had no effect on the incidence of reperfusion arrhythmias. HOE 10^{-7}M decreased the incidence of reperfusion VT from 80%

(controls) to 14% ($p<0.05$) and VF from 60% to 0% ($p<0.05$). With 10^{-6}M pretreatment, the incidence of VT was decreased from 80% to 0% ($p<0.05$) and the incidence of VF decreased from 40% to 0% (p=ns). Although not significant, 10^{-7} and 10^{-6}M HOE decreased the average time in VF from 70.7±46.1 sec to 0 sec at both concentrations (Figure 6.2).

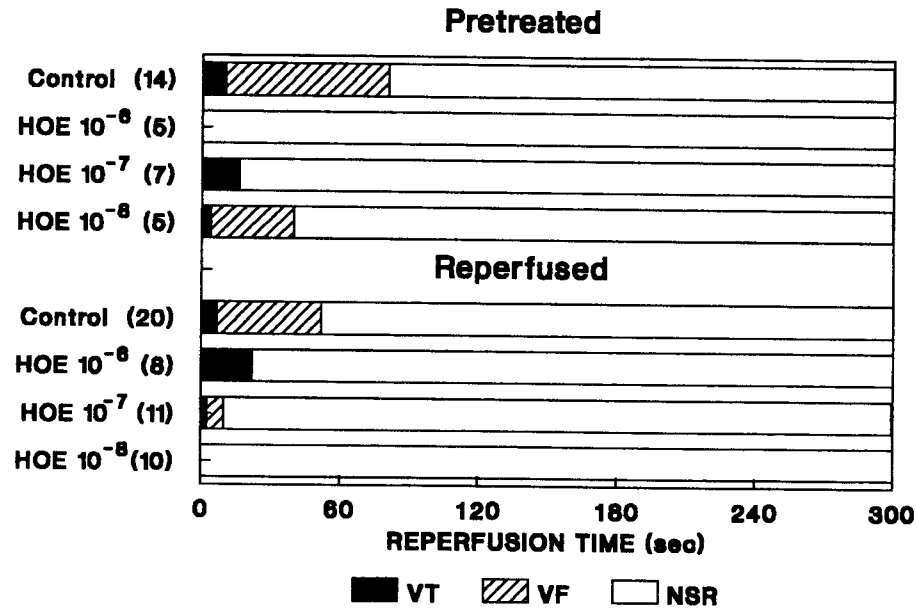

FIGURE 6.2 *Horizontal histogram showing average time in reperfusion ventricular tachycardia (VT) and/or ventricular fibrillation (VF) for groups of hearts pretreated (for 5 minutes before ischaemia) or reperfused (for initial 2 minutes) with vehicle (control) or one of three concentrations of HOE (10^{-6} to 10^{-8}M). In order to simplify figures, control data was pooled.*
( )= number of hearts per group. *HOE=HOE 694.*
VT=Ventricular tachycardia. *VF=Ventricular fibrillation.*
NSR=Normal sinus rhythm.
** $p<0.05$ vs control.* *** $p<0.002$ vs control.*

Amiloride. Two concentrations of amiloride were selected to serve as a reference for the new compound we tested. We used 10^{-3}M, effective in protecting from reperfusion stunning the Langendorff perfused rat heart (Tani

and Neely, 1989). We also used amiloride 10^{-5}M, which is anti-arrhythmic in the Langendorff model (Dennis *et al.*, 1990). Pretreatment of the working heart with 10^{-3}M amiloride did not protect the heart against reperfusion stunning (Figure 6.3).

Only the lower concentration of amiloride (10^{-5}M) given as pretreatment was effective in decreasing stunning. After 30 minutes reperfusion, AO recoveries for 10^{-5}M were 41.6$\pm$2.7% (6) for control and 55.8$\pm$4.0% (6) ($p<0.05$) for treated hearts.

Only the higher concentration of amiloride (10^{-3}M) given as pretreatment was effective in decreasing the incidence of reperfusion VT and VF. Both amiloride concentrations used decreased the average time in VT and VF but did not eliminate it completely (Figure 6.4 and Table 6.1).

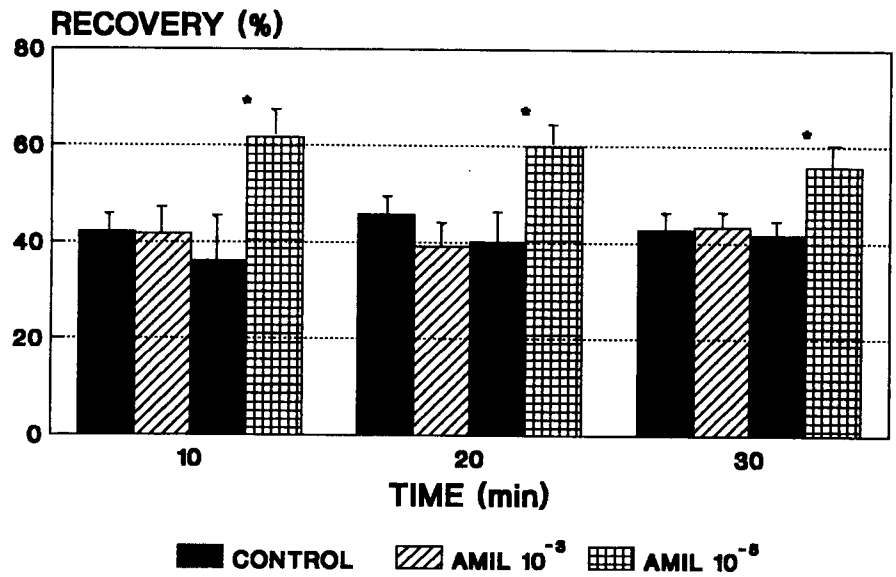

FIGURE 6.3 *Histogram showing aortic output (AO) recoveries (as a percentage of the pre-ischaemic value) after 10, 20 and 30 minutes reperfusion of hearts pretreated with amiloride $10^{-3}M$ or $10^{-5}M$ and their control groups.*
AMIL = Amiloride.
** $p<0.05$ vs control.*

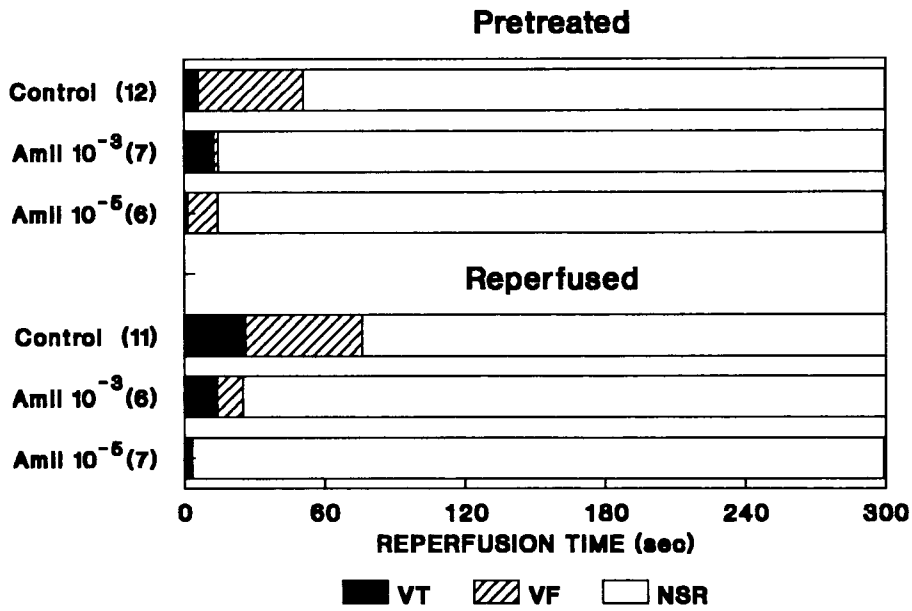

FIGURE 6.4 *Horizontal histogram showing average time in ventricular tachycardia (VT) and/or ventricular fibrillation (VF) for groups of hearts pretreated (for 5 minutes before ischaemia) or reperfused (initial 2 minutes) with vehicle (control) or one of two concentrations of amiloride (10^{-3} or 10^{-5}M). In order to simplify figures, control data was pooled.*

( )= number of hearts in the group.

AMIL=Amiloride.

VT=Ventricular tachycardia.

VF=Ventricular fibrillation.

NSR=Normal sinus rhythm.

TABLE 6.1 Incidence of reperfusion arrhythmias (VT and VF) after pretreatment of the working heart with amiloride or HOE 496 for 5 minutes before the onset of ischaemia.

Pretreatment	VT	VF
Control	4/5	4/5
Amiloride 10^{-3}M	1/7*	0/7*
Control	5/6	4/6
Amiloride 10^{-5}M	2/6*	1/6
Control	4/5	2/5
HOE 10^{-6}M	0/5	0/5
Control	4/5	2/5
HOE 10^{-7}M	1/7*	0/7
Control	3/4	3/4
HOE 10^{-8}M	2/5*	1/5

* $p<0.05$
VT=Ventricular tachycardia.
VF=Ventricular fibrillation.
Incidence expressed as a number of hearts developing the arrhythmia out of the total number of hearts perfused.

2) Effect of HOE 694 and amiloride added during the reperfusion period in hearts with left atrial cannulation.

HOE 694. The same three concentrations of HOE were used in the reperfusion solutions. At 10^{-7}M, AO recoveries were 42.4±3.8% (6) for control hearts and 65.4±5.5% (11)($p<0.05$) for treated hearts after 10 minutes reperfusion, 41.0±1.3% for control hearts and 67.1±3.7% ($p<0.002$) for treated hearts after 20 minutes, and 36.4±1.2% for control and 62.4±3.2% ($p<0.002$) for treated hearts after 30 minutes reperfusion (Figure 6.5).

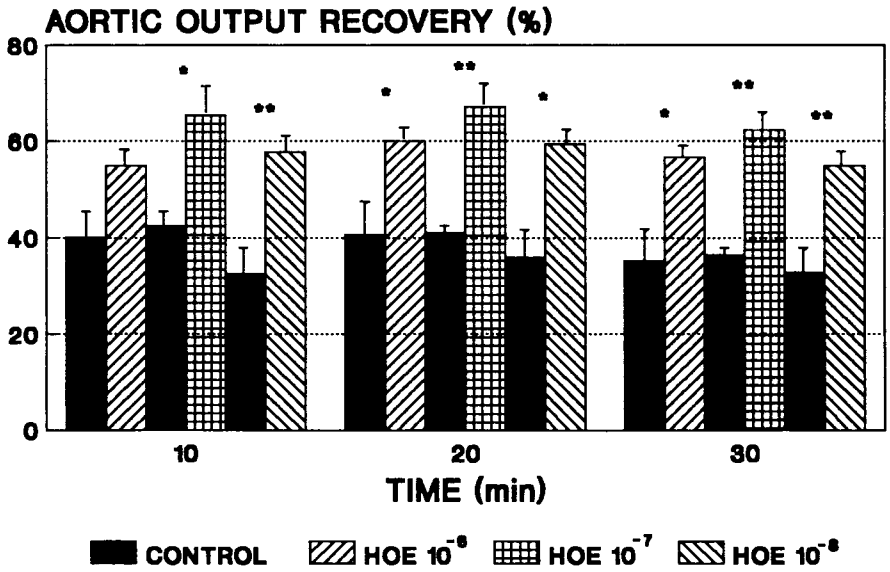

FIGURE 6.5 *Histogram showing aortic output (AO) recoveries (as a percentage of the pre-ischaemic value) after 10, 20 and 30 minutes reperfusion with HOE 694 (10^{-6} to 10^{-8}M) added only during the initial 2 minutes of reperfusion phase.*
HOE=HOE 694.
** $p<0.05$ vs control.*
*** $p<0.002$ vs control.*

HOE reperfusion decreased the incidence of reperfusion VF and VT in the treated hearts when compared with the controls. The most effective concentrations were 10^{-7} and 10^{-8}M (Figure 6.2). HOE reperfusion also decreased the average time in VF dramatically compared with the control group.

Amiloride. Reperfusion with amiloride (10^{-3}M or 10^{-5}M) offered no protection against reperfusion stunning in the working heart but 10^{-5}M did decrease the incidence of reperfusion VT from 100 to 28% ($p<0.05$) and VF from 60% to 0%

($p<0.05$). Average time in VF was also reduced by both amiloride concentrations used in reperfusion (Figure 6.4 and Table 6.2).

TABLE 6.2 Incidence of reperfusion arrhythmias during post-ischaemic reperfusion of working heart using either one of the Na^+/H^+ exchange inhibitors in the reperfusion period only (first 2 minutes).

Reperfusion	VT	VF
Control	4/6	3/6
Amiloride 10^{-3}M	2/6	1/6
Control	5/5	3/5
Amiloride 10^{-5}M	2/7*	0/7*
Control	6/6	3/6
HOE 10^{-6}M	5/8	0/8
Control	6/6	4/6
HOE 10^{-7}M	2/11*	1/11*
Control	7/8	4/8
HOE 10^{-8}M	0/10**	0/10*

* $p<0.05$ ** $p<0.002$
For definitions of VT, VF and incidence of arrhythmias, see Table 5.1.

3) Coronary effluent electrolyte composition: Coronary effluent electrolyte compositions were similar in control and treated hearts. During the early (first 15 sec) reperfusion phase, coronary effluent Na^+ and K^+ concentrations increased and pH values were decreased but normalised within the first minute after the onset of reperfusion (Figures 6.6 and 6.7).

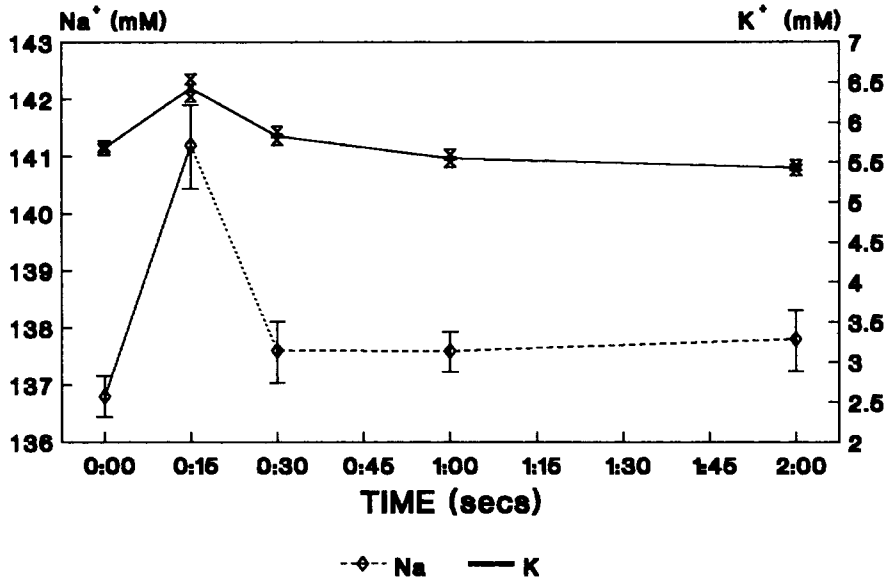

FIGURE 6.6 *Line graph showing Na^+ and K^+ efflux from hearts pretreated with HOE 694 ($10^{-6}M$). The broken line represents Na^+ and the solid line K^+ concentrations in coronary effluent during the first 2 minutes of reperfusion. Note the sharp increase in Na^+ and K^+ washout in the first 30 sec of reperfusion.*

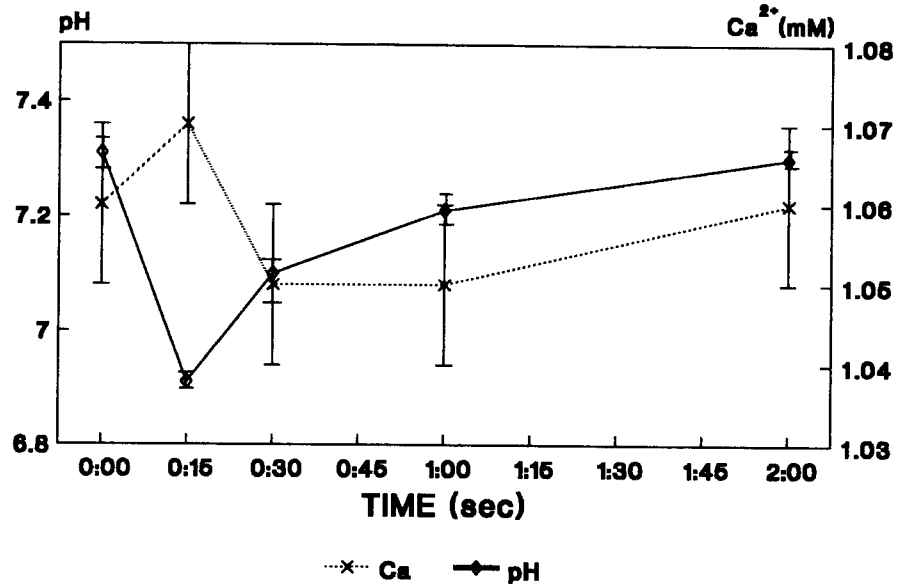

FIGURE 6.7 Line graph showing coronary effluent pH (solid line) and Ca^{2+} concentration (broken line) in the first 2 minutes of reperfusion of hearts pretreated with HOE 694 (10^{-6}M).

4) Langendorff perfused hearts and amiloride pretreatment:

Langendorff perfused hearts recovered well when pretreated with 10^{-3}M amiloride. In this case, hearts subjected to 30 minutes ischaemia were protected from reperfusion stunning. Post-ischaemic mechanical recoveries were 59 ± 1% for control and 74 ± 4% ($p<0.05$) for treated hearts after 30 minutes reperfusion (for data at 10 and 20 minutes see Figure 6.8).

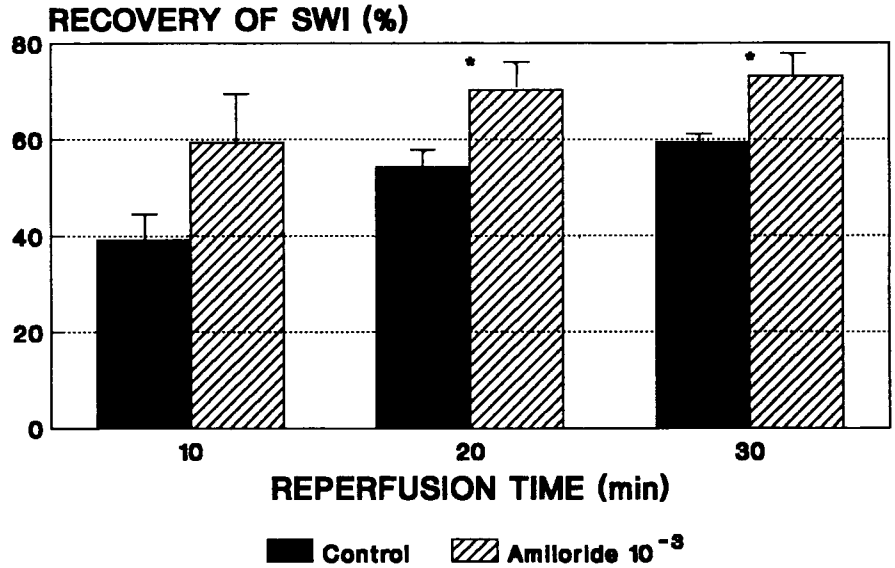

FIGURE 6.8 *Histogram showing recovery of stroke work index (SWI) after 10, 20 and 30 minutes reperfusion of Langendorff perfused hearts pretreated with amiloride ($10^{-3}M$).*
** $p<0.05$ vs control.*

Summary of the findings of study. 1) HOE 694 in very low concentrations decreased reperfusion stunning and arrhythmias in the rat heart model.

2) Activity of the Na^+/H^+ exchanger plays a role in reperfusion stunning and arrhythmias.

3) Although HOE 694 protected the working heart against reperfusion stunning either when given before ischaemia or upon reperfusion only, amiloride had no effect on stunning when given only during the reperfusion phase.

CHAPTER 7

Attenuated Reperfusion Stunning with Inhibitors of the Ca^{2+} ATP-ase Pump of the Sarcoplasmic Reticulum

a) Rationale.

While both cytosolic Ca^{2+} overload (Kusuoka *et al.*, 1987; Marban *et al.*, 1989; Opie, 1989a; Marban *et al.*, 1990; Marban 1991a; Chapter 5) and increased formation of oxygen derived free-radicals (Myers *et al.*, 1985; Przyklenk and Kloner, 1986; Bolli *et al.*, 1990) have been implicated in the genesis of reperfusion stunning, it is now recognised that these mechanisms are not mutually exclusive (Opie, 1989b; Hearse, 1991a). Therefore compounds or procedures that interfere with Ca^{2+} ion increases in the early reperfusion period may diminish reperfusion stunning. Thus far, it has been shown that, 1) increased intracellular Ca^{2+} oscillations (increased amplitude of Ca^{2+} transients) occur in reperfused, stunned hearts (Kusuoka *et al.*, 1990), and that 2) ryanodine, which inhibits abnormal intracellular Ca^{2+} oscillations (Kihara and Morgan, 1991) decreases the incidence of arrhythmias (Thandroyen *et al.*, 1988; Kihara and Morgan, 1991) and attenuates stunning (Chapter 5).

Hypothetically, part of the excess cytosolic Ca^{2+} present during ischaemia and in the early reperfusion period may originate from the sarcoplasmic reticulum. Alternatively, augmented Ca^{2+} transients found by Kusuoka *et al* (1990) may contribute to reperfusion stunning by damaging Ca^{2+} sensitive contractile organelles or by causing ATP wastage (Barry *et al.*, 1987; Kusuoka *et al.*, 1990; Benzi and Lerch, 1992) during ischaemia and early reperfusion.

Whereas in previous studies (Porterfield, Kusuoka and Weisman, 1987; Chapter 5) it was Ca^{2+} release from sarcoplasmic reticulum that was inhibited, in the

present study we inhibited uptake of Ca^{2+} into the sarcoplasmic reticulum. We used two novel inhibitors of the sarcoplasmic reticulum Ca^{2+} ATP-ase pump, namely, cyclopiazonic acid (Lahouratate, Quiniou and Leoty, 1992; Pery-man *et al.*, 1993) and thapsigargin (Thastrup *et al.*, 1990; Kirby *et al.*, 1992) to inhibit the uptake of cytosolic Ca^{2+} into the sarcoplasmic reticulum. Although most of the published data characterising these two compounds has been done on isolated SR vesicles, recent data shows that these compounds are also effective in blocking the Ca^{2+} ATP-ase pump in multicellular rabbit cardiac preparation (Baudet, Shaoulian and Bers, 1993). We propose that inhibition of the Ca^{2+} ATP-ase pump may deplete the sarcoplasmic reticulum of Ca^{2+} and/or thereby decrease intracellular Ca^{2+} oscillations, and may improve post-ischaemic mechanical function.

b) Experimental procedures, materials and methods

i) Model used: Isolated perfused working rat hearts were perfused with a standard Krebs-Henseleit buffer subject to global ischaemia and reperfusion.

ii) Experimental protocols.

1) Protocol for mechanical function studies: To determine reperfusion function after global ischaemia, the working heart model was used.

Hearts were subjected to 20 minutes total global ischaemia at a myocardial temperature of $35.5\pm0.5^{o}C$ before being reperfused in the Langendorff mode for 5 minutes. Hearts were pre-treated or reperfused with CPA (10^{-6}-10^{-8}M) or thapsigargin (10^{-6} or $2.5x10^{-8}$M). Pre-treated hearts were perfused with either compound for 3 minutes before ischaemia was initiated while hearts reperfused with the compounds were reperfused with the compound for 2 minutes at the onset of reperfusion in the Langendorff mode before normal drug-free perfusion

was initiated for a further 3 minutes. Hearts that developed reperfusion ventricular fibrillation were excluded as ventricular fibrillation causes post-fibrillation contractile dysfunction (Koretsune and Marban, 1989).

2) Ischaemic contracture study: In a separate series of experiments, hearts were perfused in the Langendorff mode, made to work for 10 minutes and pre-ischaemic mechanical function determined. At the end of the pre-ischaemic work period, a left ventricular balloon was inserted into the ventricle via the left atrium (Qiu and Hearse, 1992) and inflated to a diastolic pressure of 4-8 mmHg. Hearts were now pretreated with either vehicle, or one of the two compounds used in this study. The hearts were then made ischaemic, and diastolic pressure monitored to determine the time to onset, and amplitude of ischaemic contracture. At the end of the 20 minutes ischaemia, hearts were clamped with pre-cooled Wollenberger tongs and stored at -70^{o}C until biochemical assays were performed on the tissue samples.

iii) Metabolic profile: For details see chapter 4.

iv) Expression of data: Amplitude of ischaemic contracture was presented as a percentage (%) of the pre-ischaemic left ventricular developed pressure value. Time to onset of contracture was determined by measuring the time from the onset of ischaemia to the time when the ischaemic diastolic pressure had increased by 2 mmHg (Qiu and Hearse, 1992).

v) Pharmacologic Agents: Cyclopiazonic acid was obtained from Sigma Chemicals (St Louis, Missouri, USA). CPA was dissolved in dimethyl sulfoxide (DMSO).

Thapsigargin was obtained from LC Chemical Corporation (USA). The compound was also dissolved in DMSO and the stock was diluted to the appropriate concentration for use. The final DMSO concentration (infused into the heart) was 0.1% for 10^{-6} M thapsigargin and 0.002% for $2.5x10^{-8}$M thapsigargin.

vi) Cyclopiazonic acid and thapsigargin dose response in the non-ischaemic heart: The dose response of the working rat heart to these compounds was determined by perfusing a separate series of working hearts with a perfusion medium containing 10^{-8}, 10^{-7}, and 10^{-6} moles of thapsigargin or CPA per liter of KH buffer. The different concentrations of the inhibitor (either CPA or thapsigargin) were added successively to the same hearts and aortic output, coronary flows and left ventricular pressures were measured after 10 minutes of perfusion at each concentration.

c) Results

1) Cyclopiazonic acid and thapsigargin dose response in the non-ischaemic heart.

Cyclopiazonic acid decreased aortic output and coronary flow in a dose dependent manner in the non-ischaemic working heart when infused in the micro to nanomolar concentration range (Figure 7.1). Control aortic output values in the CPA group were 41.2±2.3 ml/min and fell to 87±2% of the control value after a 10 minutes perfusion with 10^{-8}M CPA. Aortic output values were further reduced to 76±3% ($p<0.05$) of the control value after a 10 minutes perfusion with 10^{-7}M CPA and to 63±5% ($p<0.002$) after a 10 minute perfusion with 10^{-6}M CPA.

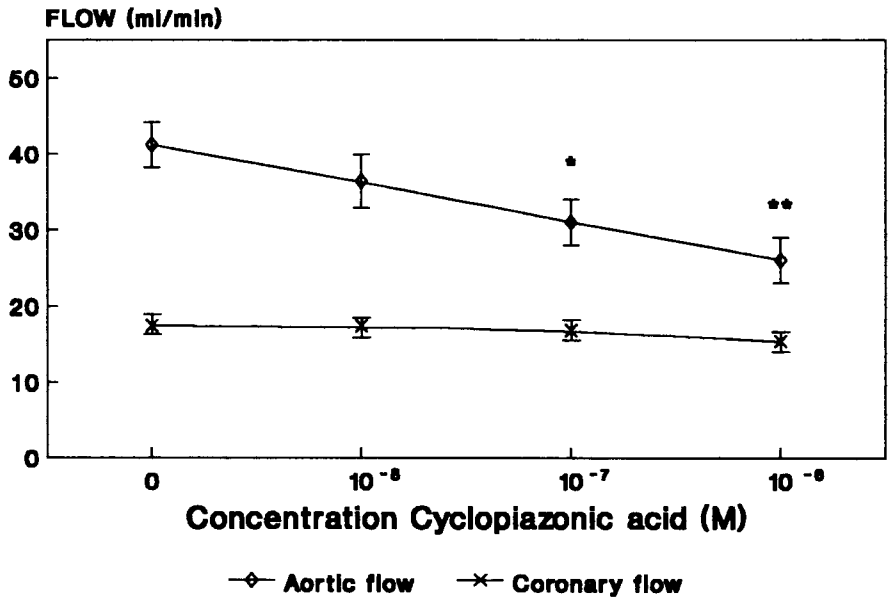

FIGURE 7.1 *Dose responses curve for cyclopiazonic acid in the isolated working non-ischaemic rat heart. The compound was infused for 10 minutes at each concentration and aortic and coronary flows measured. After these measurements were completed, the next concentration was administered.*

* $p<0.05$ *vs control.*

** $p<0.002$ *vs control*

Thapsgargin elicited a weak negative inotropic response with control hearts generating aortic output values of 46.2±4.4 ml/min. After a 10 minutes perfusion with 10^{-8}M thapsigargin AO values decreased to 96±1% of the control value. A further 10 minutes perfusion with 10^{-7}M decreased aortic output to 92±1% of the control value and 10 minutes perfusion with 10^{-6}M caused a fall to 81±4% of the control value (Figure 7.2).

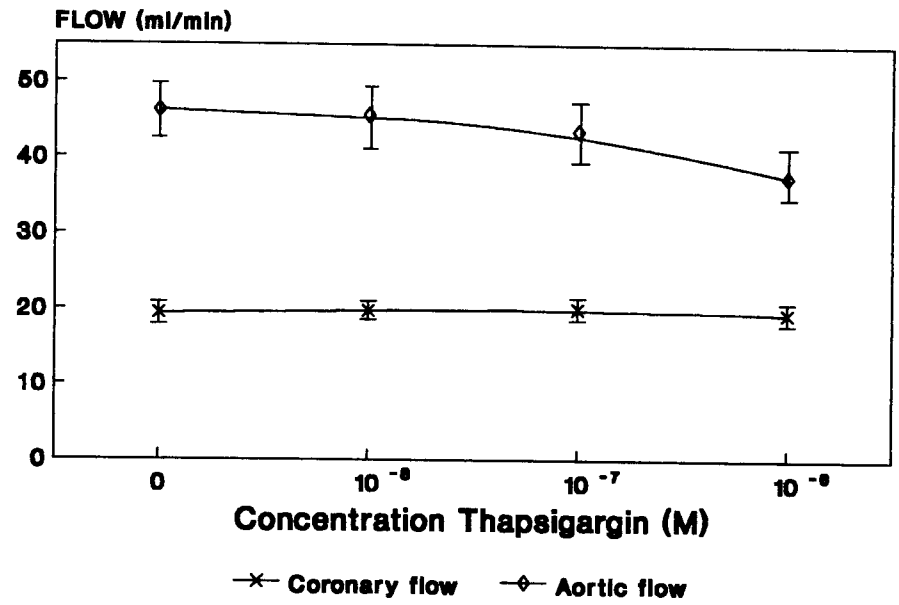

FIGURE 7.2 Dose responses curve for thapsigargin in the isolated working non-ischaemic rat heart. The compound was infused for 10 minutes at each concentration and aortic flow and coronary flow was measured. After these measurements were completed, the next concentration was administered.

I. Mechanical recoveries after global ischaemia.

A. Pre-treatment with CPA (Figure 7.3): Hearts pre-treated with CPA (10^{-6} and 10^{-7}M) had better aortic output recoveries than the control group. The lower concentration of CPA (10^{-8}M) had no effect on reperfusion function, resulting in reperfusion AO recoveries of 67.4±2.2% (n=5) compared to control values of 73.7±2.6% (n=7) after 15 minutes work.

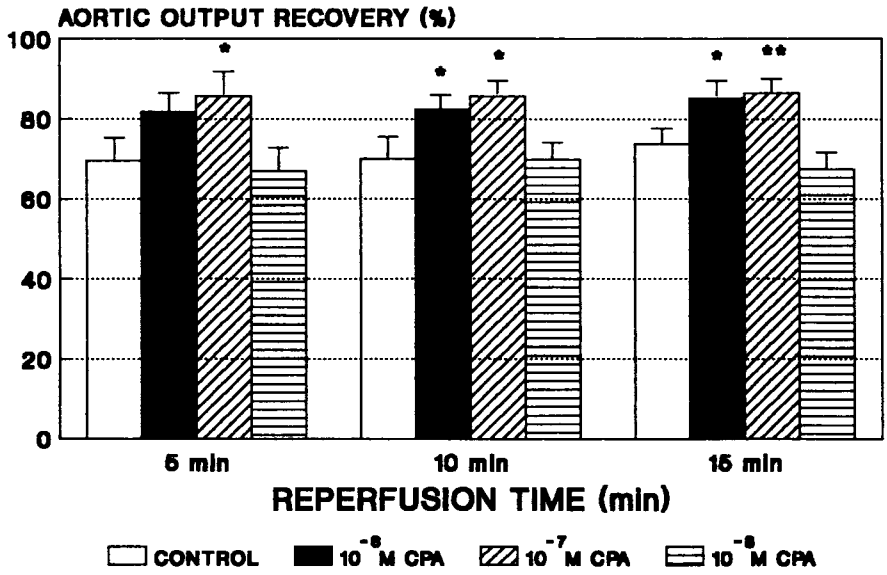

FIGURE 7.3 *Reperfusion aortic output recoveries of hearts pre-treated with vehicle (control) or one of three concentrations of cyclopiazonic acid (CPA) (10^{-6}-10^{-8}M).*

* *p<0.05 vs control.*

** *p<0.002 vs control.*

Pre-treatment with the optimal CPA concentration (10^{-7}M) (Figure 7.4) before ischaemia gave aortic output (AO) values of 42.1±1.3 ml/min (7) before ischaemia and 39.4±1.9 ml/min after 15 min work during reperfusion. This corresponds to an AO recovery of 93.3±1.6% compared to 78.2±3.0% ($p<0.002$) for control untreated hearts. Pre-ischaemic coronary flows for treated hearts were 15.1±0.5 ml/min and reperfusion values were 15.0±0.5 ml/min (at 15 minutes work). Control hearts had AO of 44.9±1.1 ml/min (12) before ischaemia and 35.2±1.6 ml/min after ischaemia. There were no differences in coronary flows before and after ischaemia in the control group (Table 7.1).

TABLE 7.1 Reperfusion function of the working rat heart before and after 20 minutes global ischaemia with CPA pretreatment (Pre) or CPA at the time of reperfusion (Rep). Data is presented as means ± standard error of the mean.

	Pre-ischaemia				Reperfusion (15min)			
	CF	**AO**	**DP**	**SP**	**CF**	**AO**	**DP**	**SP**
Control	16.0	44.9	17	117	16.1	35.2	17	108
	0.7	1.1	0	1	0.8	1.6	0	2
CPA Pre	15.1	42.1	17	115	15.2	39.4	17	114
	0.4	1.3	0	2	0.5	1.9	1	2
CPA Rep	17.2	45.3	16	114	17.5	40 9	16	110
	0.8	1.5	0	3	0.6	2.0	0	2

AO = Aortic output.
CF = Coronary flow.
DP = Diastolic pressure.
SP = Systolic pressure.

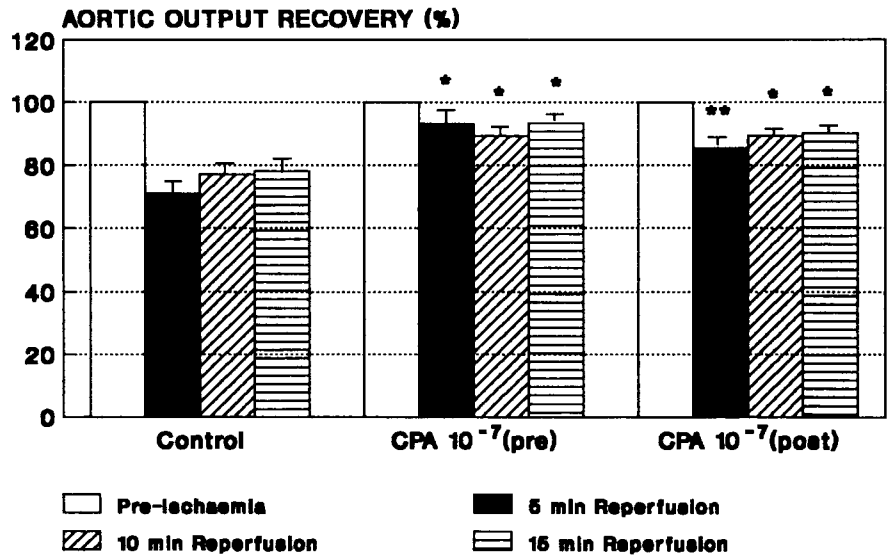

FIGURE 7.4 *Aortic output recoveries (at 5, 10 and 15 minutes reperfusion) of hearts pretreated before 20 minutes global ischaemia (pre), or reperfused with cyclopiazonic acid (CPA) (10^{-7}M)(post) or vehicle (control).*

* *$p<0.05$ vs control.*

** *$p<0.002$ vs control.*

Pre-treatment with thapsigargin. (Figure 7.5): Pretreatment with thapsigargin (2.5×10^{-8}M) protected the heart against reperfusion stunning. AO values for control hearts were 45.1 ± 1.0 ml/min before ischaemia and 28.4 ± 2.1 ml/min (6) at 5 minutes work after ischaemia. In hearts pretreated by thapsigargin, the mean reperfusion aortic output value improved to 44.4 ± 4.4 ml/min (6) after ischaemia (Table 7.2) compared with the reperfusion value of 28.5 ± 3.7 ml/min in controls.

Hearts pre-treated with higher doses of thapsigargin (10^{-6}M) had AO outputs of 44.0 ± 3.2 ml/min (8) before ischaemia and AO of 3.0 ± 1.5 ml/min 10 minutes after reperfusion. Of eight hearts pre-treated, only 3 hearts generated any AO

after ischaemia. Even these 3 hearts had totally failed by the 15 minutes work period. Coronary flow values after ischaemia were decreased with pre-ischaemic values of 18.7±0.8 ml/min and reperfusion values of 11.4±1.9 ml/min (at 10 minutes work). Control hearts had AO of 44.6±1.8 ml/min (14) before ischaemia and 33.9±2.1 ml/min after ischaemia. There were no differences in coronary flow values before and after ischaemia in the control group.

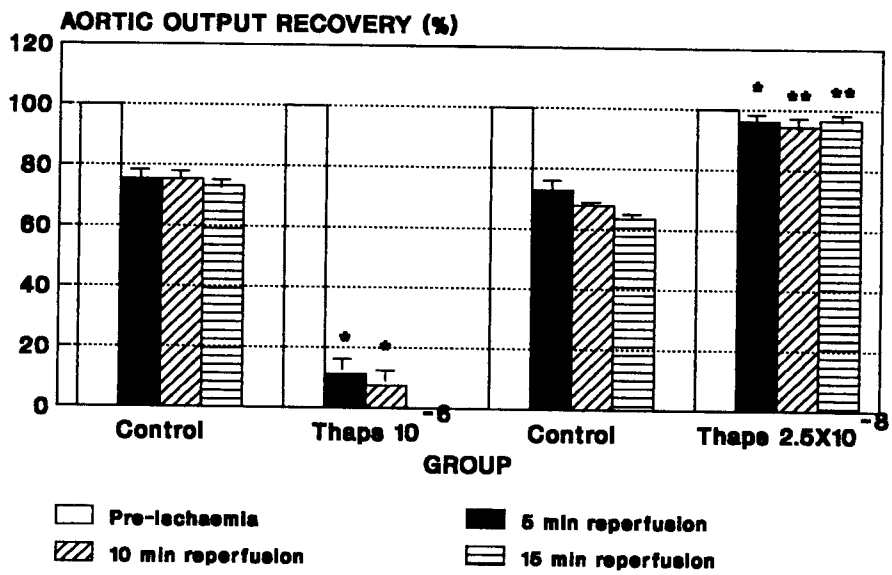

FIGURE 7.5 *Aortic output recoveries (at 5, 10 and 15 minutes reperfusion) of hearts pretreated with thapsigargin (Thaps) (10^{-6} or $2.5x10^{-8}$ M) or with vehicle (control) before 20 minutes global ischaemia.*

* *$p<0.05$ vs control.*

** *$p<0.002$ vs control.*

TABLE 7.2 Reperfusion function of the working rat heart before and after 20 minutes global ischaemia with thapsigargin (Thaps) ($2.5X10^{-8}M$) pretreatment (Pre) or thapsigargin at the time of reperfusion (Rep). Data is presented as means ± standard error of the mean.

	Pre-ischaemia				Reperfusion(15min)			
	CF	AO	DP	SP	CF	AO	DP	SP
Control	18.9	45.1	11	99	16.6	28.4	12	87
	0.4	1.0	1	2	0.8	2.1	2	2
Thaps Pre	17.3	45.8	13	111	18.3	44.8	14	104
	1.1	2.7	3	4	0.8	3.7	4	2
Thaps Rep	19.4	48.5	11	105	19.3	38.0	11	95
	1.0	1.9	1	3	1.5	3.2	1	2

AO = Aortic output.
CF = Coronary flow.
DP = Diastolic pressure.
SP = Systolic pressure.

B. Reperfusion with CPA: Pre-ischaemic aortic outputs of hearts treated with CPA ($10^{-7}M$) during reperfusion were 45.3±1.5 ml/min and that of control hearts were 44.9±1.1 ml/min. The reperfusion function recoveries (%) for CPA hearts, were 85.8±1.5% after 5 minutes work, 89.3±1.5% after 10 minutes, and stabilised at 90.2±2.7% after 15 minutes work. The control hearts had AO recoveries of 71.2±2.4% after 5 minutes work, 77.3±3.1% after 10 minutes and 78.2±3.0% (12) after 15 minutes (Figure 7.4). Coronary flows were 17.2±0.8 for the treated group and 16.8±0.7 ml/min for the control group. The post-ischaemic coronary flow values for these two groups were unchanged.

Reperfusion with thapsigargin (Figure 7.6): Reperfusion with ($2.5x10^{-8}M$) thapsigargin improved early reperfusion AO recovery. Pre-ischaemic values were 48.5±1.9 ml/min and reperfusion values 38.0±3.8 ml/min (6) after 15 minutes work.

Reperfusion with high thapsigargin concentrations ($10^{-6}M$) improved early reperfusion function (86.7±3.0% at 5 minutes reperfusion) but caused a rapid decline in function towards 15 minutes reperfusion (61.4±5.0%). Thapsigargin reperfusion had no effect on post-ischaemic coronary flows.

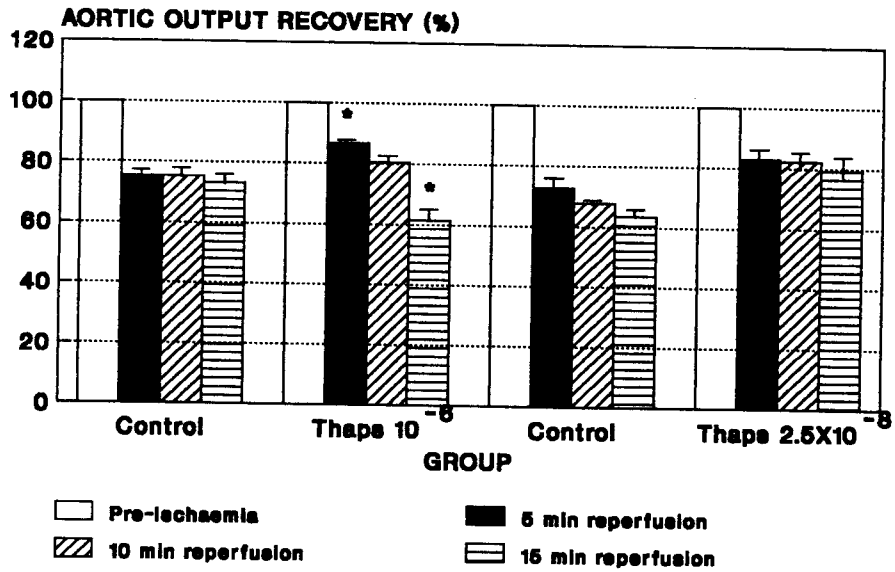

FIGURE 7.6 *Aortic output recoveries (at 5, 10 and 15 minutes reperfusion) of hearts made globally ischaemic for 20 minutes and reperfused with thapsigargin (Thaps) ($10^{-6}M$ or $2.5x10^{-8}M$) for 2 minutes.*

* *$p<0.05$ vs control.* ** *$p<0.002$ vs control.*

II. Cyclopiazonic acid, thapsigargin, and ischaemic contracture (Figure 7.7).

Pre-treatment of the isolated rat heart with CPA or thapsigargin had no effect on the time to onset of contracture, but did influence the magnitude of contracture. Pretreatment with CPA (10^{-7}M) or thapsigargin ($2.5x10^{-8}$M) decreased the % contracture to 8.1±3.9% (n=6) and 3.7±3.7% ($p<0.05$) (n=6) respectively compared to a control value of 23.4±7.7% (n=8). High concentrations of thapsigargin (10^{-6}M) increased the magnitude of ischaemic contracture to 33.6±5.4% compared with both CPA (10^{-7}M) (8.1±3.9%)($p<0.05$) and thapsigargin ($2.5x10^{-8}$M) (3.7±3.7%) ($p<0.05$).

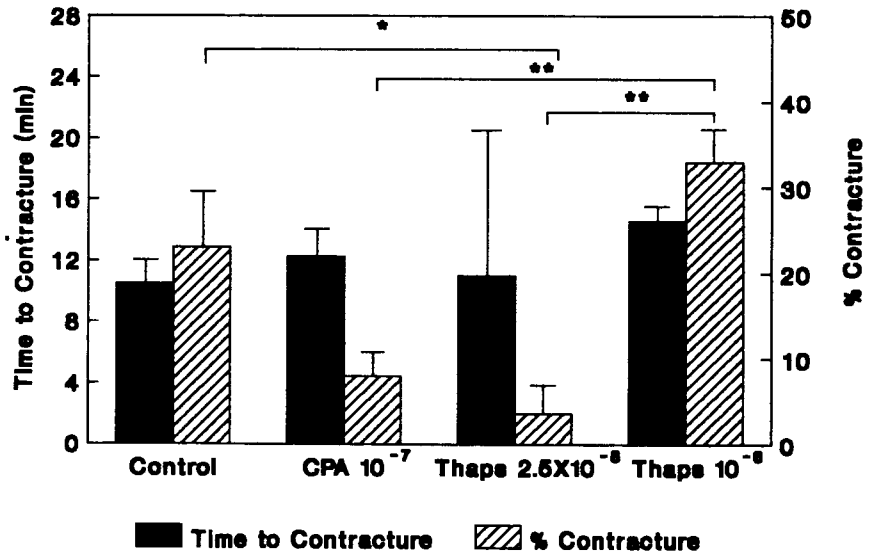

FIGURE 7.7 Histogram presenting data for the time to onset of, and magnitude of ischaemic contracture for hearts pretreated with CPA (10^{-7}M), thapsigargin (10^{-6} or $2.5x10^{-8}$M), or vehicle (control).

* *$p<0.05$ vs control.*

** *$p<0.05$ vs Thaps (10^{-6}M).*

III. Cyclopiazonic acid, thapsigargin, and post-ischaemic metabolic status of pre-treated hearts (Figure 7.8).

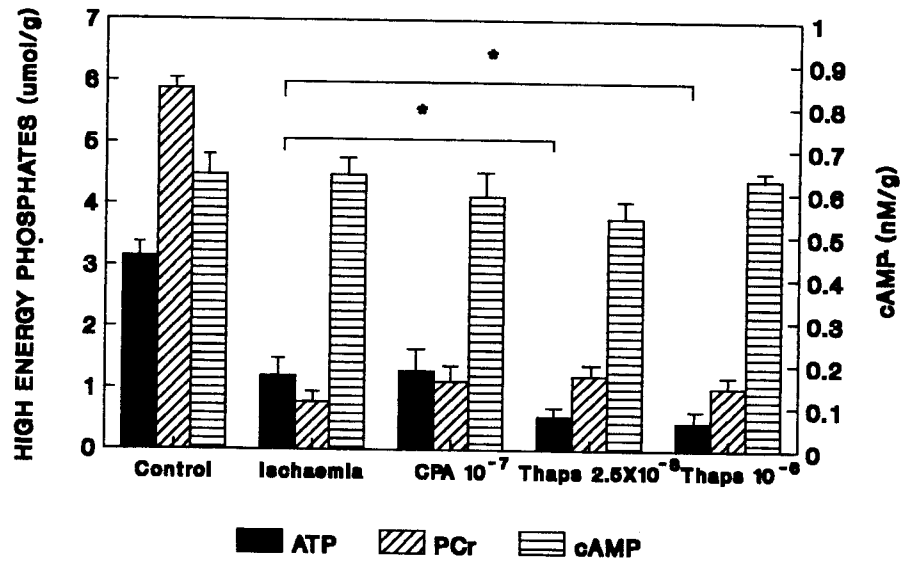

FIGURE 7.8 *Histogram presenting tissue ATP, PCr, and cAMP levels at the end of 20 minutes ischaemia in control and drug pre-treated hearts.*
* *$p<0.05$ vs Ischaemia group.*

Pretreatment with the ATP-ase pump inhibitors had no effect on the end ischaemic cAMP or PCr levels. Both thapsigargin concentrations ($2.5x10^{-8}$ and 10^{-6}M) decreased the tissue ATP levels at the end of ischaemia. Control post-ischaemic ATP levels were 1.2 ± 0.26 μmol/g (n=8) compared with 0.5 ± 0.05 μmol/g ($p<0.05$)(n=6) and 0.45 ± 0.11 μmol/g ($p<0.05$)(n=6) respectively. There were no differences in tissue cAMP levels between control and drug pre-treated groups.

Summary of findings of study. 1) Inhibition of Ca^{2+} ATP-ase pump of the sarcoplasmic reticulum (SR) before ischaemia or during reperfusion protected the isolated working rat heart against reperfusion stunning.

2) These findings support the proposal that the SR may contribute to reperfusion stunning by i) being a source of excess cytosolic Ca^{2+} during ischaemia and reperfusion (Kusuoka *et al.*, 1990) and/or, ii) by contributing to augmented intracellular Ca^{2+} transients (oscillations) which may damage the contractile apparatus or contribute to ATP depletion in the stunned heart (Barry *et al.*, 1987; Benzi and Lerch, 1992).

CHAPTER 8

Thapsigargin and Cyclopiazonic acid: Antiarrhythmic Properties of Specific Inhibitors of the Sarcoplasmic Reticulum Ca^{2+} ATP-ase Pump after Coronary Artery Ligation.

a) Rationale.

Despite many recent advances in the treatment of ischaemic heart disease, sudden cardiac death associated with ventricular fibrillation remains a major cause of mortality in Western society (Suravicz, 1985). Recently the proposal has been made that excess Ca^{2+} oscillations may play an important role in ischaemic ventricular fibrillation (For a review, see Lubbe *et al.*, 1992). Oscillatory release of Ca^{2+} from intracellular stores is a primary event underlying changes in the transient inward current which contributes to arrhythmogenic activity (Kass *et al.*, 1978; Kass, Tsien and Weingart, 1978).

Hypothetically, such oscillations, if magnified, could play an important role in the genesis of ventricular arrhythmias both during ischaemia and reperfusion. Ryanodine and caffeine, both acting on the sarcoplasmic reticulum to modulate intracellular Ca^{2+} fluxes, have antiarrhythmic properties (Thandroyen *et al.*, 1988; Kihara and Morgan, 1991). If Ca^{2+} oscillations from the sarcoplasmic reticulum play a major role in the initiation or perpetuation of serious ventricular arrhythmias, then specific inhibition of the metabolic pump taking up Ca^{2+} from the cytosol into the sarcoplasmic reticulum would also be expected to be antiarrhythmic.

Ischaemic and reperfusion arrhythmias were provoked in the isolated rat heart model by coronary artery ligation and release (reperfusion). Thapsigargin, which is thought to be very specific, and inhibits 60-70% of the Ca^{2+} ATP-ase pumps

in isolated cardiac sarcoplasmic reticulum (Thastrup *et al.*, 1990; Sagara and Inesi, 1991; Kirby *et al.*, 1992), was infused into regionally ischaemic hearts and the incidence of ischaemic and reperfusion arrhythmias was monitored. In a separate series of experiments, a second specific and possibly more potent Ca^{2+} ATP-ase pump inhibitor, cyclopiazonic acid (Seidler *et al.*, 1989), was used.

b) Experimental procedures, materials and methods.

i) Model Used: Male Long-Evans rat hearts perfused in the Langendorff mode were used for all perfusions.

ii) Perfusion fluid: The Krebs-Henseleit perfusion buffer used for the ischaemic arrhythmia study was modified to contain a potassium concentration of 4.8 mM. For the reperfusion arrhythmia study, the Ca^{2+} concentration was 2.5 mM to increase the incidence of reperfusion arrhythmias under control conditions (Coetzee *et al.*, 1990). The buffers were gassed with 95%O_2 and 5%CO_2, and maintained at 37^{o}C.

The left anterior descending coronary artery was ligated, electrocardiograph (ECG) electrodes were attached to the aortic cannula and the right ventricle and the incidence of ischaemic and reperfusion arrhythmias were monitored by means of an ECG recording (for details see Chapter 4).

iii) Protocols used.

A. Protocol for ischaemic arrhythmia study.

1) Hearts were allowed a 10 minutes equilibration period before the drug or vehicle (DMSO 0.01-0.1%) infusion was initiated and maintained for the duration of the experiment. After 5 minutes treatment the left anterior

descending coronary artery was ligated and drug infusion rate adjusted to the new (post-ligation) coronary flow rate (Figure 8.1).

2) In a second series of hearts, hearts were allowed a 10 minutes equilibration period, the coronary artery was ligated, and drug or vehicle infusion was initiated 5 minutes after ligation. The ligation was left in position for 30 minutes and the coronary flow, heart rate, left ventricular pressure (monitored with a balloon) and ECGs were monitored until the end of the 30 minutes ischaemic period.

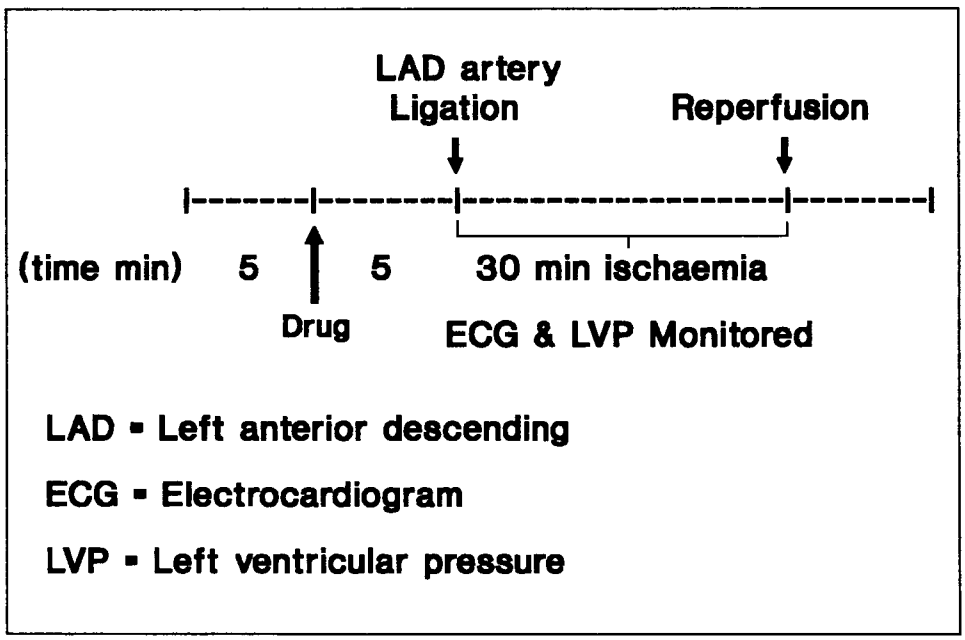

FIGURE 8.1 *Diagrammatic representation of the protocol used for the ischaemic arrhythmia study. Drug infusion was initiated 5 minutes before or after (not indicated in figure) coronary artery ligation and the incidence of ischaemic arrhythmias monitored for the 30 minutes of coronary artery ligation.*

B. Protocol for reperfusion arrhythmia study.

Hearts were stabilized for 10 minutes before the drug or vehicle infusion was initiated. After 5 minutes infusion, left anterior descending coronary artery ligation was employed to induce regional ischaemia for 8, 10, 15, 20, 25 or 30

minutes. Reperfusion was achieved by cutting the ligature across a polyvinyl sheath which encompassed the coronary artery. Coronary flow, heart rate, and ECG were again recorded throughout the experiment and for the first 2 minutes of reperfusion (Figure 8.2).

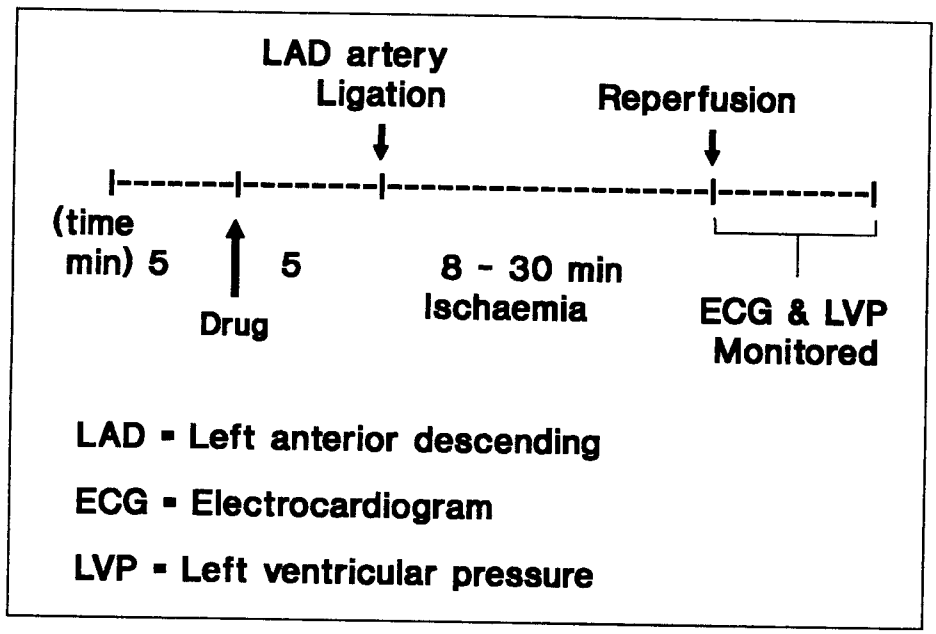

FIGURE 8.2 *Diagrammatic representation of the experimental protocol used for the reperfusion arrhythmia study.*

iv) Metabolic profile.

Hearts were freeze-clamped for later biochemical analysis using Wollenberger tongs pre-cooled in liquid nitrogen, after 15, 20 or 30 minutes of ischaemia for tissue high energy phosphate determinations. Adenosine triphosphate (ATP) and phosphocreatine (PCr) were assayed spectrophotometrically at 340 nm in samples from ischaemic and non-ischaemic myocardium (for details pertaining to assay methods, see chapter 4).

v) Evaluation of Ventricular Arrhythmias.

For a definition of arrhythmias in this model, see chapter 4.

vi) Mechanical function studies.

Left ventricular pressure was monitored using left ventricular cannulation (20G Abbocath-T catheter) or an ultrathin "Clingfilm" balloon inserted into the left ventricle (Tani and Neely, 1989; Qiu and Hearse, 1992). The cannula or balloon was attached to a Stratham Gould P23ID pressure transducer and the left ventricular developed pressure and ECG was recorded with a Lectromed chart recorder.

Coronary flow and heart rates were monitored throughout the experiment.

vii) Pharmacologic Agents.

Thapsigargin and cyclopiazonic acid was prepared as in chapter 7. The two doses (10^{-6}M thapsigargin and 10^{-7}M cyclopiazonic acid) used for these arrhythmia studies were chosen to have similar negative inotropic effects, both decreasing aortic flow by about 15-20% (See chapter 7).

c) Results

1) Arrhythmias and Thapsigargin.

A. Ischaemic ventricular arrhythmias: (Figure 8.3) The incidence of ventricular fibrillation in control hearts was 83% while in the treated hearts it was 0% ($p<0.002$). The duration of ventricular fibrillation was 389.6 ± 102 sec for control hearts (12) and 0 sec for treated hearts (12) ($p<0.05$). The duration of ventricular tachycardia and the number of ventricular premature beats was similar for control and treated hearts.

Thapsigargin treatment initiated 5 minutes after coronary artery ligation decreased the incidence of ischaemic ventricular fibrillation from 77% (control) (9) to 14% (7) ($p<0.05$).

There were no difference in the coronary flow values or heart rates between control and the thapsigargin treated hearts.

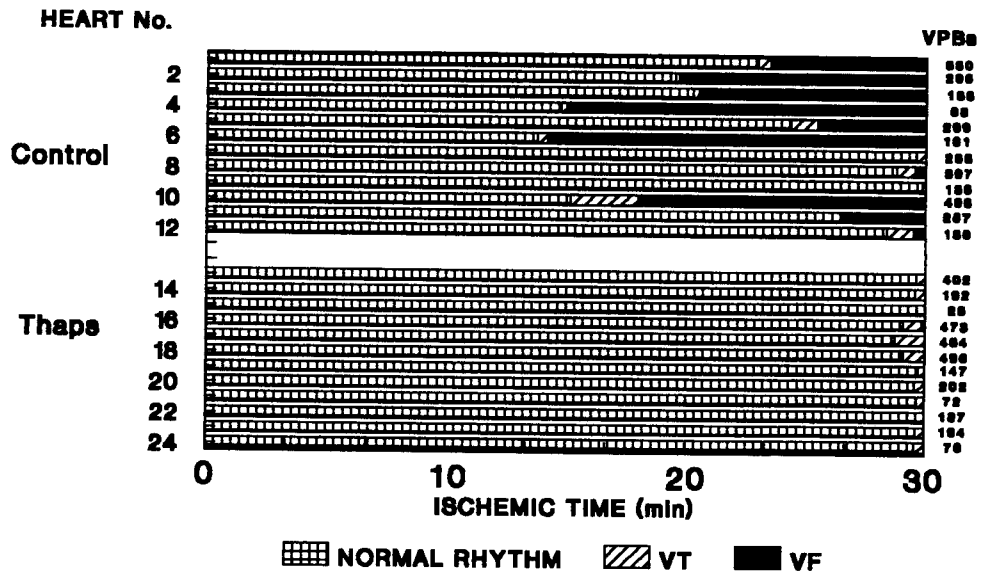

FIGURE 8.3 ***Ischaemic arrhythmias.*** *Horizontal histogram presenting the absolute time (in minutes) of the duration of ischaemic ventricular tachycardia or fibrillation in individual Langendorff perfused hearts during 30 minutes ischaemia. Control hearts are represented by the top 12 bars while thapsigargin treated hearts are represented by the bottom 12 bars. The number of ventricular premature beats (VPBs) are presented to the right of each bar. Note major antiarrhythmic action of thapsigargin.*

VT= Ventricular tachycardia.

VF= Ventricular fibrillation.

Thaps = Thapsigargin $10^{-6}M$.

Post-ischaemic levels of adenosine triphosphate and phosphocreatine were measured in the ischaemic and non-ischaemic zones. Tissue ATP levels in the ischaemic zone were lower than those from the non-ischaemic zone in all hearts except in thapsigargin treated hearts after 15 and 20 minutes ischaemic. ATP levels were 2.40 ± 0.14 μmol/g in the ischaemic tissue and 2.83 ± 0.15 μmol/g in the non-ischaemic tissue of thapsigargin treated hearts after 15 minutes ischaemic, and 2.30 ± 0.18 μmol/g and 2.78 ± 0.30 μmol/g respectively after 20 minutes ischaemic. The absence of a tissue gradient for ATP coincided with a decrease in the incidence of reperfusion arrhythmias at 15 and 20 minutes reperfusion in the thapsigargin treated groups (Table 8.1).

TABLE 8.1 ATP and PCr values after 15, 20 and 30 minutes ischaemia in the ischaemic and non-ischaemic region of control, Thaps (thapsigargin) and CPA (cyclopiazonic acid) treated hearts.

	ATP (μmol/g)	PCr (μmol/g)
NONLIGATED HEARTS(n=16)	3.83±0.09	5.07±0.17
15 min ISCHAEMIA		
CONTROLS(n=7)		
Ischaemic	2.12±0.21	1.57±0.3
Non-ischaemic	3.24±0.16*	3.05±0.20**
THAPS HEARTS(n=7)		
Ischaemic	2.40±0.14	0.98±0.14
Non-ischaemic	2.83±0.15	2.43±0.26**
CPA HEARTS(n=6)		
Ischaemic	2.09±0.20	1.12±0.26
Non-ischaemic	3.39±0.21*	2.77±0.36*
20 min ISCHAEMIA		
CONTROLS(n=7)		
Ischaemic	1.67±0.20	1.33±0.23
Non-ischaemic	3.17±0.17*	3.24±0.24**
THAPS HEARTS(n=7)		
Ischaemic	2.30±0.18	1.69±0.21
Non-ischaemic	2.78±0.30	2.41±0.44
CPA HEARTS(n=7)		
Ischaemic	2.34±0.23	1.95±0.21
Non-ischaemic	3.36±0.23*	3.31±0.59
30 min ISCHAEMIA		
CONTROLS(n=7)		
Ischaemic	2.03±0.26	1.77±0.19
Non-ischaemic	3.63±0.24*	2.63±0.50
THAPS HEARTS(n=6)		
Ischaemic	2.12±0.17	1.43±0.17
Non-ischaemic	3.28±0.19*	1.86±0.42
CPA HEARTS(n=7)		
Ischaemic	1.77±0.19	1.51±0.43
Non-ischaemic	3.31±0.10*	3.16±0.20*

* $p<0.05$ vs non-ischaemic myocardium.
** $p<0.002$ vs non-ischaemic myocardium.

B. Reperfusion ventricular arrhythmias: There was a direct correlation between the incidence of reperfusion arrhythmias and the ischaemic time, up to about 20 minutes. The incidence of reperfusion ventricular fibrillation increased from 29 to 67, 81 and 90% in control hearts subjected to 8, 10, 15 or 20 minutes ischaemia respectively. After 25 and 30 minutes there was a decrease to 56% (9) and 45% (11) respectively. With thapsigargin (10^{-6}M) treatment, the incidence of reperfusion ventricular fibrillation was reduced to 0, 0 ($p<0.05$), 25 ($p<0.002$), 46 ($p<0.05$), 38 and 27% respectively (Figure 8.4). During reperfusion of the 10 and 15 minutes ischaemia groups, the incidence of reperfusion ventricular tachycardia was reduced from 83 to 0% ($p<0.002$) and 87 to 45% ($p<0.05$) respectively (Figure 8.5). Table 8.2 presents absolute values for time in VF and VT (of groups) under control and drug treatment conditions.

These differences in the incidence of reperfusion arrhythmias occurred without any significant difference in the heart rate or coronary flow of the treated compared with the control hearts (data not shown).

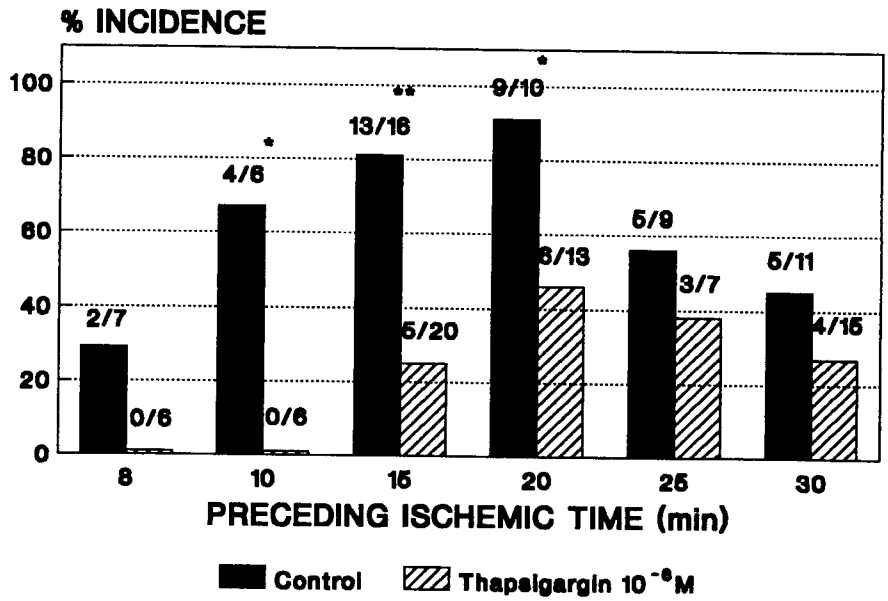

FIGURE 8.4 ***Reperfusion ventricular fibrillation.*** *Histogram presenting the percentage incidence of reperfusion ventricular fibrillation in control and thapsigargin treated Langendorff perfused hearts after 8, 10, 15, 20, 25 or 30 minutes of regional ischaemia.*

For additional information, see Table 8.2.

** $p<0.05$ vs control.*

*** $p<0.002$ vs control.*

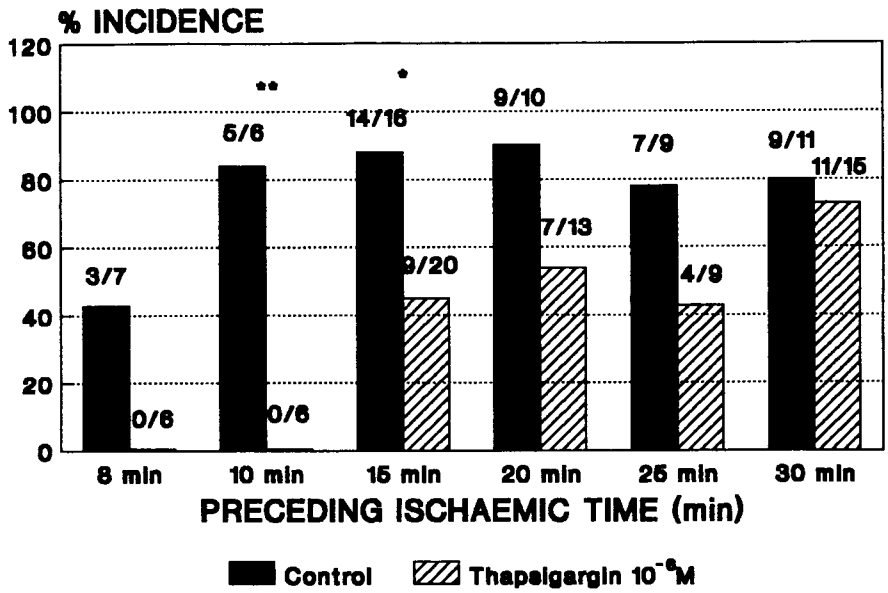

FIGURE 8.5 ***Reperfusion ventricular tachycardia.*** *Histogram presenting the percentage incidence of reperfusion ventricular tachycardia in control and thapsigargin treated Langendorff perfused hearts after 8, 10, 15, 20, 25 or 30 minutes of regional ischaemia.*

** $p<0.05$ vs control.*

TABLE 8.2 Reperfusion arrhythmias showing absolute values and durations for control hearts and hearts perfused with thapsigargin (10^{-6}M).

Ischaemic time	n	VPBs (no)	VT time (sec)	VF
10 min				
Control	6	25±8	8±3	72±19
Thapsigargin	6	3±0.4 *	0 *	0 *
15 min				
Control	16	9±1	8±1	95±8
Thapsigargin	20	6±1 *	12±2	89±15
20 min				
Control	10	10±5	8±2	99±11
Thapsigargin	13	11+3	7±1	103±2
25 min				
Control	9	21±6	9±4	71±17
Thapsigargin	7	20±4	7±3	61±26
30 min				
Control	10	10±4	18±7	58±15
Thapsigargin	15	18±4	8±1	72±12

Control hearts were perfused with DMSO 0.1%.
n = number of hearts done per group.
VPBs = Ventricular premature beats (presented as an absolute value).
VT = Ventricular tachycardia and ventricular fibrillation (VF) is presented as a time (seconds).
* $p<0.05$ vs controls.

2) Arrhythmias and Cyclopiazonic acid.

A. Ischaemic ventricular arrhythmias (Figure 8.6): The incidence of ventricular fibrillation in control hearts was 83% while the incidence in the treated hearts was 16% ($p<0.05$). The duration of ventricular fibrillation was 101.7±7.8 sec for control hearts (6) and 120 sec for treated hearts (only one heart developed ventricular fibrillation). The time in ventricular tachycardia was 259.5±84.6 sec (6) for control hearts and 47.6±31.7 sec (6) ($p<0.05$) for cyclopiazonic acid treated hearts. Only one treated heart developed ventricular fibrillation while five control hearts fibrillated.

Treatment by cyclopiazonic acid initiated 5 minutes after coronary artery ligation decreased the incidence of ischaemic ventricular fibrillation from 77% (control) (9) to 0% (8) ($p<0.05$).

Ischaemic values for coronary flow and heart rate were unchanged by cyclopiazonic acid treatment.

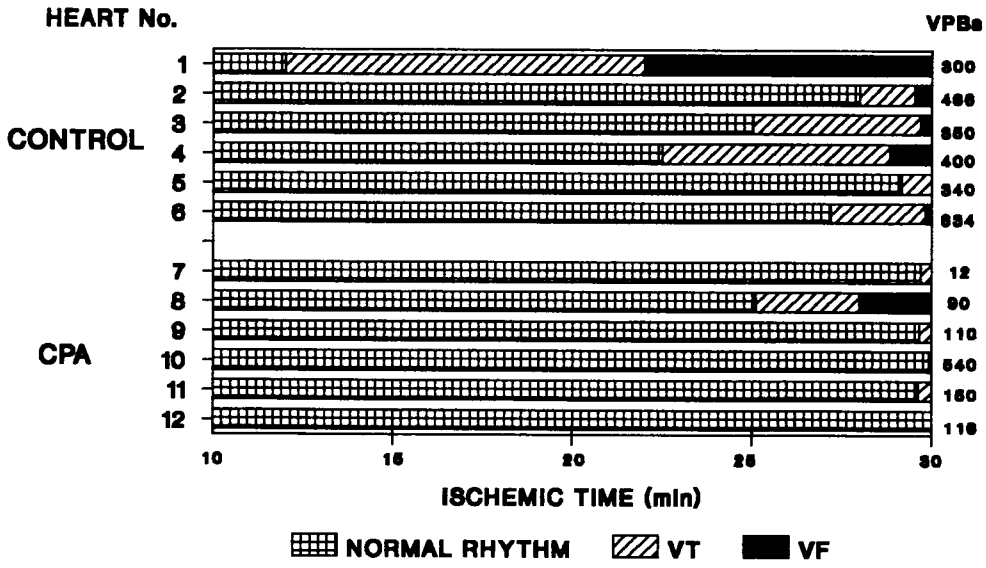

FIGURE 8.6 Ischaemic arrhythmias. Control hearts are represented by the top 6 bars while cyclopiazonic acid (CPA) treated hearts are represented by the bottom 6 bars. For further details, see legend to Figure 8.3.

B. Reperfusion ventricular arrhythmias: The incidence of reperfusion ventricular fibrillation increased from 50, to 83, to 100% in control hearts subjected to 10, 15 or 20 minutes of ischaemia. At 25 and 30 minutes there was a decrease in the incidence of ventricular fibrillation to 71 and 62% respectively. With cyclopiazonic acid (10^{-7}M) treatment the incidence of reperfusion ventricular fibrillation was reduced to 33, 40, 14 ($p<0.05$), 44, and 50% respectively (Figure 8.7). These differences in the incidence of ventricular fibrillation occurred without any differences in the heart rate or coronary flow of the treated compared with the control hearts.

The number of ventricular premature beats was consistently higher in the cyclopiazonic acid treated hearts when compared with controls (Table 8.3).

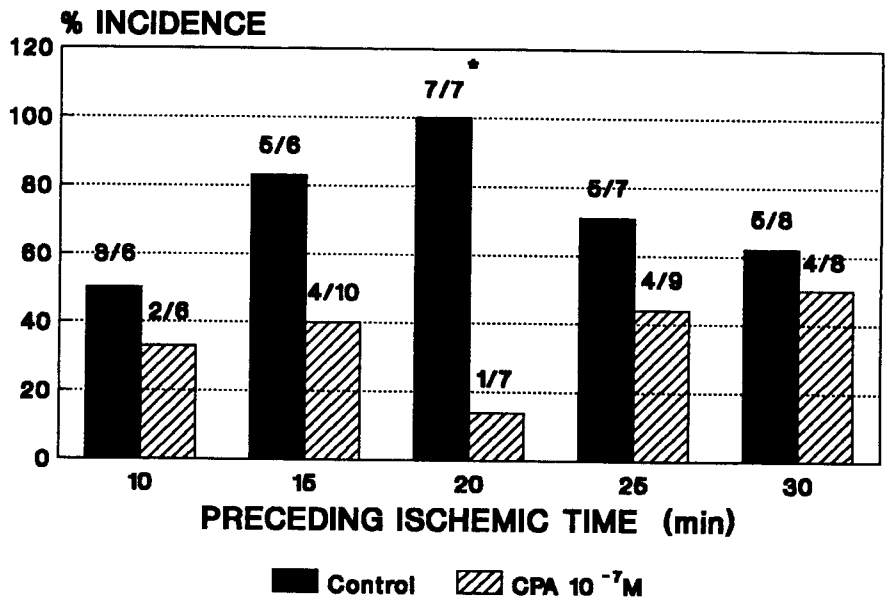

FIGURE 8.7 ***Reperfusion ventricular fibrillation.*** *The incidence percentage of reperfusion ventricular fibrillation in control and cyclopiazonic acid treated Langendorff hearts subject to 10, 15, 20, 25 and 30 minutes ischaemia. For additional information, see Table 8.2.*
CPA = Cyclopiazonic acid $10^{-7}M$.
** $p<0.05$ vs control.*

TABLE 8.3 Reperfusion arrhythmias presented as absolute values (VPBs) and durations (VT and VF) for control hearts and hearts perfused with cyclopiazonic acid (CPA) (10^{-7}M).

Ischaemic time	n	VPBs (no)	VT time (sec)	VF time (sec)
10 min				
Control	6	8±1	8±2	73±22
CPA	6	14±3	9±1	72±17
15 min				
Control	6	7±3	11±3	103±4
CPA	10	15±5	15±2	60±14*
20 min				
Control	7	11±3	44±4	97±3
CPA	7	29±8	4±0.2**	56±0**
25 min				
Control	7	20±7	9±3	94±6
CPA	9	32±6	8±2	61±18
30 min				
Control	8	36±6	14±7	72±14
CPA	8	33±6	23±9	90±15

Control hearts were perfused with DMSO 0.01%.
n = the number of hearts per group.
VPBs = Ventricular premature beats (presented as absolute values).
VT = Ventricular tachycardia and ventricular fibrillation (VF) is presented as a time (seconds).
* $p<0.05$ vs controls.
** $p<0.002$ vs controls.

3) Mechanical Function during Coronary Artery Ligation.

Coronary artery ligation decreased the rate-pressure product of all hearts by ±60%. Rate-pressure product values of the drug treated hearts increased over the next 25 minutes and were 14508±675 (thaps) (7) ($p<0.05$) and 14636±1365 (CPA) (8) ($p<0.05$) compared with 10859±663 (9) for controls (Figure 8.8).

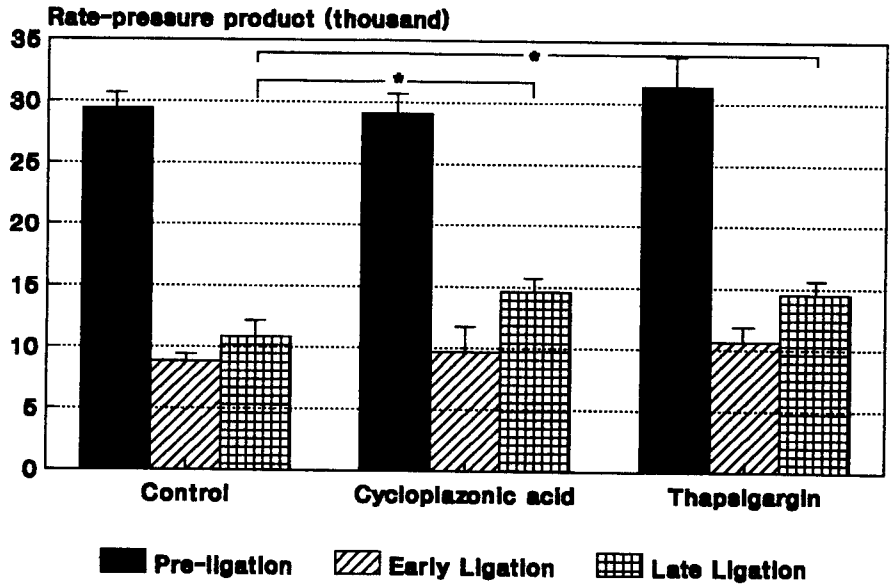

FIGURE 8.8 ***Rate-pressure product before and during coronary artery ligation.*** *The rate-pressure product for control and drug treated hearts are presented. The solid bar represents the rate-pressure product before coronary artery ligation, the cross-hatched bar the value 5 minutes after ligation and the dotted bar the value 25 minutes after drug or vehicle treatment was initiated.*

** $p<0.05$ vs control.*

Summary of findings of study. 1) Excess internal Ca^{2+} ion oscillations play an important role in the genesis and perpetuation of ischaemic and reperfusion ventricular fibrillation.

2) The use of two specific inhibitors (Seidler *et al.*, 1989; Kirby *et al.*, 1992) of uptake of Ca^{2+} ions into the sarcoplasmic reticulum, decreased the incidence of spontaneous ischaemic and reperfusion ventricular arrhythmias.

CHAPTER 9

Endothelin Release during Ischaemia and Reperfusion and its Role in Reperfusion Injury.

a) Rationale.

Endothelin is an endogenous 21-amino-acid peptide with multiple biological actions (Yanagisawa *et al.*, 1988). It contracts vascular and non-vascular smooth muscle in vitro, produces a sustained pressor response in vivo and exerts a positive inotropic effect on atrial and ventricular cardiac muscle (for recent reviews see Lerman *et al.*, 1990; Nayler, 1990; Simonson and Dunn, 1990; Rubanyi and Parker-Botelho, 1991).

Endothelin may play an important cardiovascular regulatory and/or pathophysiological role. When endothelin was injected into the coronary circulation of isolated rabbit hearts, dose-dependent vasoconstriction, increased outflow of prostacyclin and impaired diastolic relaxation was observed (Karwatowska-Prokopczuk and Wennmalm, 1990). Furthermore, endothelin, by virtue of its ability to mobilize intracellular Ca^{2+} (Vigne *et al.*, 1990), could hypothetically contribute to cytosolic Ca^{2+} overloading of cardiomyocytes, and therefore reperfusion injury (Opie, 1989a; Nayler, 1990). In rat cardiac membranes, ischaemia causes time-dependent externalization of endothelin-1 binding sites (Liu, Casley and Nayler, 1989), thus increasing the number of receptor sites available for the interaction with blood-borne endothelin. A possible role for endothelin in myocardial infarction is suggested because circulating levels of endothelin increased within hours of onset of myocardial infarction (or presentation) in humans (Miyauchi *et al.*, 1989, Salminen *et al.*, 1989, Yasuda *et al.*, 1990, Stewart *et al.*, 1991). An endothelin-antibody reduced experimentally-induced infarct size in the rat heart (Watanabe *et al.*, 1991).

The hypothesis tested in this study was that endogenous endothelin plays a role in events associated with or leading to myocardial ischaemia and/or post-ischaemic reperfusion damage (Nayler, 1990). Several models of ischaemia were used to test whether endogenous endothelin was released into the coronary circulation. The influence of exogenous endothelin on post-ischaemic coronary perfusion pressure and recovery of reperfusion mechanical function (reperfusion vascular and myocardial stunning, respectively) was also studied.

b) Experimental procedures, materials and methods.

i) Model Used: For all functional and endothelin-release studies, rat hearts perfused with a Krebs-Henseleit buffer on the isolated working rat heart and Langendorff perfusion system were used (for details see chapter 4).

ii) Experimental protocol: Four experimental protocols were used in this study.

Protocol 1: Endothelin release during ischaemia and reperfusion.

To determine whether endogenous endothelin is released during ischaemia and/or reperfusion, hearts were perfused in the Langendorff mode at a constant pressure of 100 cm H_2O for 15 minutes (equilibration period). They were then subjected to different models of ischaemia and reperfused under constant pressure for up to 60 minutes. Coronary effluent samples were collected at the end of the equilibration period (10 to 15 minutes after mounting), during ischaemia and, depending on the model, from 0 to 5, 5 to 30 and 30 to 60 minutes of reperfusion.

Model 1: For this model, low flow ischaemia for 90 or 180 minutes was used (Owen *et al.*, 1990). Hearts were perfused at a constant flow rate of 0.5-0.6 ml/min with the standard perfusion medium and the coronary effluent collected

quantitatively in one (90 minutes) or two (180 minutes) sample(s). During reperfusion (5-60 minutes), coronary effluent was recirculated.

In a variation of this model, the perfusates used during ischaemia contained 1 % foetal calf serum.

Model 2: In the second model, global ischaemia for various periods of time was used (Chapter 5). Total ischaemia was induced by shutting off aortic flow for 20 or 30 minutes, hence no coronary effluent was obtained during ischaemia in this case. The temperature was maintained between 35 and 36^{O}C. Reperfusion was in the non-recirculating (20 minutes ischaemia) or recirculating mode (30 minutes ischaemia). In the latter case, aprotinin (6 K IU/ml) was added to the perfusate.

Protocol 2: Effect of endothelin on coronary perfusion pressure and left ventricular systolic pressure in control hearts.

Rat hearts (approximate weight 0.9-1.1 g) were perfused in the Langendorff mode until a stable coronary flow, about 8 to 11 ml/min, was obtained. The left ventricle was cannulated with a teflon cannula (Abbocath-T), connected to a pressure transducer and left ventricular systolic pressure was continuously recorded. Coronary perfusion pressure was measured with a second transducer at a point in the aortic cannula approximately 3 cm above the heart. After an initial equilibration period (about 15 minutes) under constant pressure, constant flow perfusion was initiated at the flow rate equivalent to that obtained at the end of the (constant pressure) equilibration period, thus preventing the development of myocardial ischaemia due to endothelin-induced vasoconstriction. After stabilisation of the left ventricular systolic and coronary perfusion pressure traces, endothelin-2 (1 ng (0.4 pmol) to 1 μg (400 pmol) was slowly injected by cumulative dosing into the perfusion medium directly above the heart in volumes

between 0.1 and 0.3 ml at 2.5 minute intervals (Fukuda *et al.*, 1989). A perfusion medium containing 0.1% bovine serum albumin (BSA) was used to check the effect of injection on perfusion pressure. The vasoactive effect of endothelin on the coronary circulation was assessed from the changes in coronary perfusion pressure and the inotropic effect from the changes in maximum systolic ventricular pressure.

Protocol 3: Effect of endothelin-2 on coronary perfusion pressure during reperfusion.

Hearts were perfused retrogradely at constant pressure for 10 minutes and the coronary flow rate was determined. Hearts were then perfused at a constant flow rate until a stable baseline was obtained (pre-ischaemic control), followed by a 1-hour low-flow (0.5 ml/min) perfusion in the absence of foetal calf serum. Constant flow reperfusion at the pre-ischaemic rate was resumed, a stable pressure baseline obtained (6-11 minutes after onset of reperfusion) and endothelin-2 added cumulatively at doses ranging from 0.1 ng (0.04 pmol) to 1 μg (400 pmol). The resulting dose-response-curves were analyzed as above.

Protocol 4: Effect of endothelin on reperfusion mechanical function.

After 5 minutes of aortic perfusion in the Langendorff mode at a constant pressure of 100 cm H_2O, perfusion was changed to one of constant flow for 5 minutes at the flow rate determined at the end of the Langendorff equilibration period. This was followed by perfusion in the work mode for 10 minutes. Following the measurement of functional parameters (pre-ischaemic control), the hearts were subjected to total global ischaemia for 20 minutes before being reperfused for 5 minutes in the Langendorff mode at a constant flow rate, equal to the pre-ischaemic rate. Langendorff reperfusion was followed by 40 minutes perfusion in the working mode and a final 5 minute Langendorff perfusion under

constant pressure (73 mm Hg). The total time for each experiment was 90 minutes. The left ventricle was cannulated for pressure measurements as above. Hearts were paced at 5 Hz for the entire experiment except during ischaemia and the first 2 minutes of reperfusion.

Endothelin-2 was injected into the aorta via a T-piece as a bolus dose of 100 or 200 ng at the onset of reperfusion. The effect of endothelin on reperfusion mechanical function was assessed by measuring the aortic output prior to ischaemia and 10, 20, 30 and 40 minutes after the onset of post-ischaemic work. The results were expressed as percentage recoveries (%) of the pre-ischaemic value. The effect of endothelin-2 on aortic output of non-ischaemic control hearts (ischaemic period replaced by Langendorff perfusion) was determined for the same time points in separate experiments.

iii) Materials and methods for measurement of endothelin.

For materials and methods used for endothelin measurement see chapter 4.

c) Results

1) Basal endothelin release

The basal endothelin release rate was determined in hearts using normoxic perfusate. Due to the complete cross-reactivity of the antibody, all three endothelin species would presumably be quantified. The results are presented in Table 9.1 Endothelin release was constant up to 180 minutes and amounted to 0.69 ± 0.02 pg/min/g heart weight. Neither the heart rate nor the coronary flow changed significantly over this time period (ANOVA-1, $p>0.05$).

TABLE 9.1 Basal endothelin (ET) release from isolated rat hearts.

Sampling period (min post-mounting)	Heart rate (bpm)	Coronary flow (ml/min/g)	ET-release (pg/min/g)
10-15	310±27	7.4±0.2	0.71±0.05
25-30	318±10	7.3±0.1	0.66±0.05
55-60	285±16	7.9±1.2	0.63±0.03
85-90	321±30	7.3±1.1	0.68±0.07
115-120	297±15	6.6±1.2	0.68±0.05
145-150	288±17	6.2±0.9	0.68±0.04
175-180	264+14	5.9+1.0	0.69+0.04

Values are presented as mean ± S.E.M., n=5. The total amount of effluent collected for each period was column-chromatographed. ET release is expressed per min and g wet wt. None of the three variables measured changed significantly over the entire course of the experiment ($p>0.05$).

2) Endogenous endothelin in ischaemia and reperfusion

To determine if the isolated rat heart releases endogenous endothelin during ischaemia and after subsequent resumption of coronary perfusion, the 2 different experimental models of ischaemia (described earlier in this section) were chosen, perfusate samples collected from these models and analyzed for endothelin. Since various sera have been shown to increase stimulated endothelin-secretion of endothelial cells (Hexum *et al.*, 1990, Ristimäki *et al.*, 1991), foetal calf serum was added to the perfusate in one model. The results obtained with the low-flow model of ischaemia are presented in Figure 9.1 and Table 9.2. Compared to the basal (pre-ischaemic) secretion rate, endothelin release was greatly reduced during an ischaemic period of 90 or 180 minutes ($p<0.01$). On reperfusion (0-5 minutes), immunoreactive endothelin increased 3.9-fold ($p<0.05$) in hearts subjected to 180 minutes of ischaemia in the presence of 1% foetal calf serum while the increase was only 1.4-fold in its absence ($p>0.05$, Figure 9.1, Table 9.2). After 90 minutes of ischaemia, endothelin release rate increased non-significantly 1.4- and 1.15-fold in the presence and absence of foetal calf serum,

respectively ($p>0.05$, Table 9.2). Between 5 and 30 minutes of reperfusion the release rates declined to or below control levels.

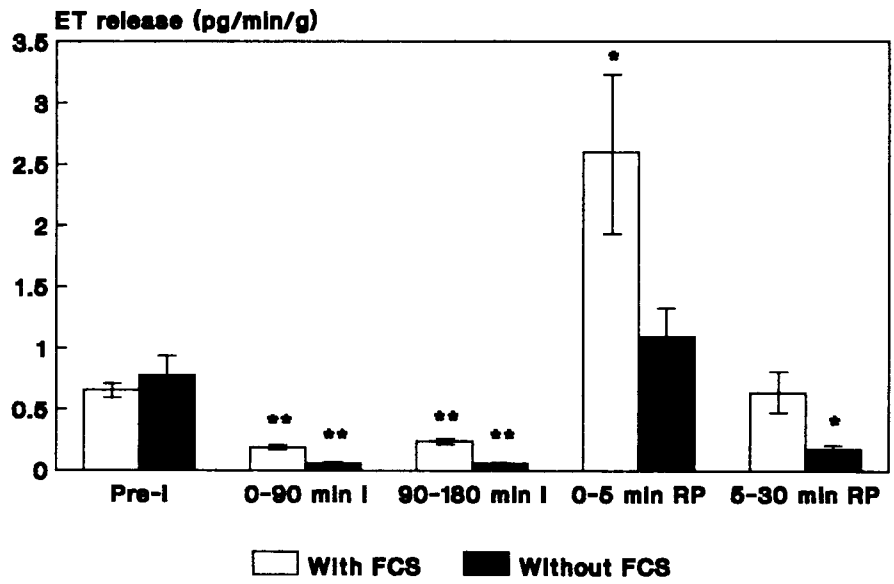

FIGURE 9.1 *Endothelin release in low-flow ischaemia and reperfusion. Hearts were perfused under control conditions, then in the absence and presence of 1% foetal calf serum (FCS) under low-flow (0.5 ml/min) hypoxic conditions for 180 minutes (I) and subsequently reperfused under normoxic conditions for 30 minutes (RP).*

Means ± S. E. M.

n=6-7.

* *$p<0.05$ vs pre-ischaemic control.*

** *$p<0.01$ vs pre-ischaemic control.*

TABLE 9.2 Endothelin (ET) release during low-flow ischaemia (I) in the presence and absence of foetal calf serum (FCS) and on reperfusion (RP).

	90 min Ischaemia			
	With 1 % FCS (n=7)		**Without FCS (n=6)**	
Sampling period	**ET release (pg/min/g)**	**Change over Pre-ischaemic control (-fold)**	**ET release (pg/min/g)**	**Change over Pre-ischaemic control (-fold)**
Pre-ischaemic control	0.93±0.13		0.74±0.10	
I 0-90	0.63±0.005[a]	0.07±0.008	0.05±0.003[a]	0.07±0.007
I 90-180	-	-	-	-
RP 0-5	1.32±0.29	1.38±0.16	0.89±0.26	1.15±0.22
RP 5-30	0.24±0.03[a]	0.27±0.04	0.17±0.01[a]	0.24±0.02.
	180 min Ischaemia			
	With 1 % FCS (n=7)		**Without FCS (n=6)**	
Sampling period	**ET release (pg/min/g)**	**Change over Pre-ischaemic control (-fold)**	**ET release (pg/min/g)**	**Change over Pre-ischaemic control (-fold)**
Pre-ischaemic control	0.66±0.05		0.78±0.16	
I 0-90	0.19±0.02[a]	0.28±0.03	0.06±0.007[a]	0.08±0.01
I 90-180	0.24±0.02[a]	0.36±0.04	0.06±0.007[a]	0.09±0.01
RP 0-5	2.60±0.63[b]	3.94±0.92	1.10±0.23	1.44±0.15
RP 5-30	0.64±0.17	0.95±0.18	0.18±0.03[b]	0.23±0.03

Values are presented as mean ± S.E.M. The model is described under Experimental protocol. [a]$P<0.01$; [b]$P<0.05$.

In a model of 20 minutes of global ischaemia, endothelin release on reperfusion was initially also, albeit non-significantly increased (1.32-fold, $p>0.05$), similar to controls between 5 and 30 minutes, and again non-significantly increased between 30 and 60 minutes (Table 9.3). Total global ischaemia for 30 minutes resulted in release rates (0.67±0.05 pg/min/g) which were not higher than controls (0.62±0.07 pg/min/g, $p>0.05$). Thereafter, release declined below 0.15 pg/min/g ($p<0.01$, data not shown).

TABLE 9.3 Endothelin (ET) release on reperfusion (RP) subsequent to 20 minutes global ischaemia.		
Sampling period	**ET release (pg/min/g)**	**Change over control (-fold)**
Pre-ischaemic control	0.73±0.07	
RP 0-5 min	0.92±0.10	1.32±0.17
RP 10-15 min	0.69±0.07	1.04±0.21
RP 25-30 min	0.66±0.04	0.96±0.12
RP 55-60 min	0.85+0.07	1.31+0.30.
Values are presented as mean ± S.E.M., n=7. All effluent collections were made in the non-circulating mode of perfusion. No statistically significant changes were observed ($P<0.05$).		

3) Effect of endothelin-2 on the coronary perfusion pressure of non-ischaemic and ischaemic isolated rat hearts

Bolus injections of endothelin-2 provoked dose-dependent increases in coronary perfusion pressure within the range of 1ng to 1μg per heart (Figure 9.2, diamonds). The vehicle for endothelin-2 had only a transient reversible effect. The perfusion pressure increased slowly and reached a maximum after 1 to 3 min. This pressure was maintained for at least 5 minutes except when the highest doses were administered. Coronary perfusion pressure increased from 65±2.1 (baseline) to a maximum of 134±7.5 mm Hg (+ 106% increase). The threshold dose for the vasoconstrictive action was ≈20 ng, and the EC_{50} calculated by non-linear regression analysis of pooled data was 69 ng per heart.

Hearts made ischaemic through low-flow perfusion for 1 hour reacted more readily to exogenous endothelin-2 (0.1ng to 1μg) than normoxic hearts (Figure 9.2, crosses): the baseline coronary perfusion pressure was 93±2.4 mmHg and increased to a maximum of 188±4.5 mmHg (+ 238 %) at 1 μg endothelin-2. The threshold dose was below 1 ng, and the EC_{50} calculated by non-linear regression of pooled data was 1.9 ng per heart; Figure 9.2).

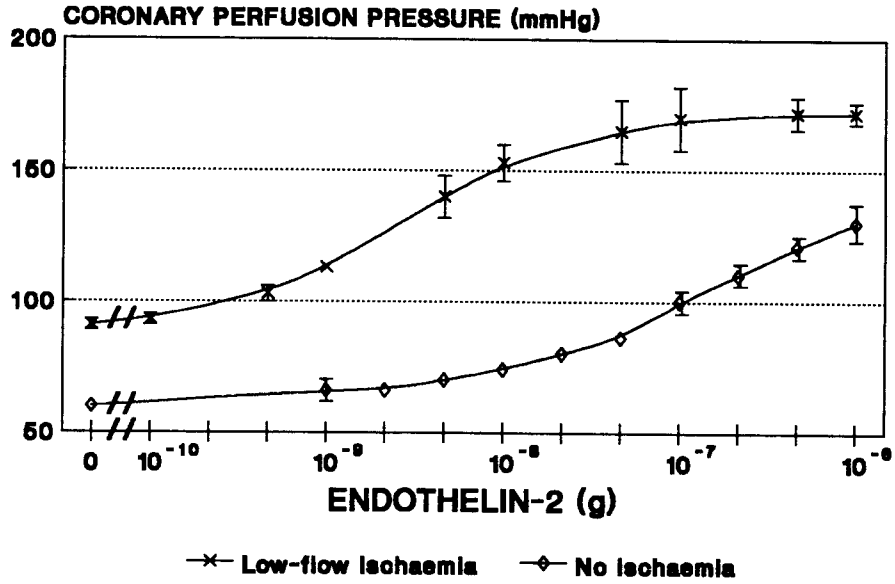

FIGURE 9.2 *Dose response curve for the effect of endothelin-2 on coronary perfusion pressure in control and reperfused hearts. Endothelin was applied to hearts perfused at constant flow without (controls, diamonds) or subsequent to 1 hour of low-flow ischaemia (reperfused hearts, crosses). Data are expressed as absolute pressure readings (mmHg) as a function of endothelin-2 dose (g per heart) applied cumulatively at 2.5 minute intervals. The mean curve was fitted by non-linear regression and the EC_{50} determined as 69 (control) and 1.9 (reperfusion) ng endothelin-2 per heart. Means $\pm$ S.E.M., $n=3$.*

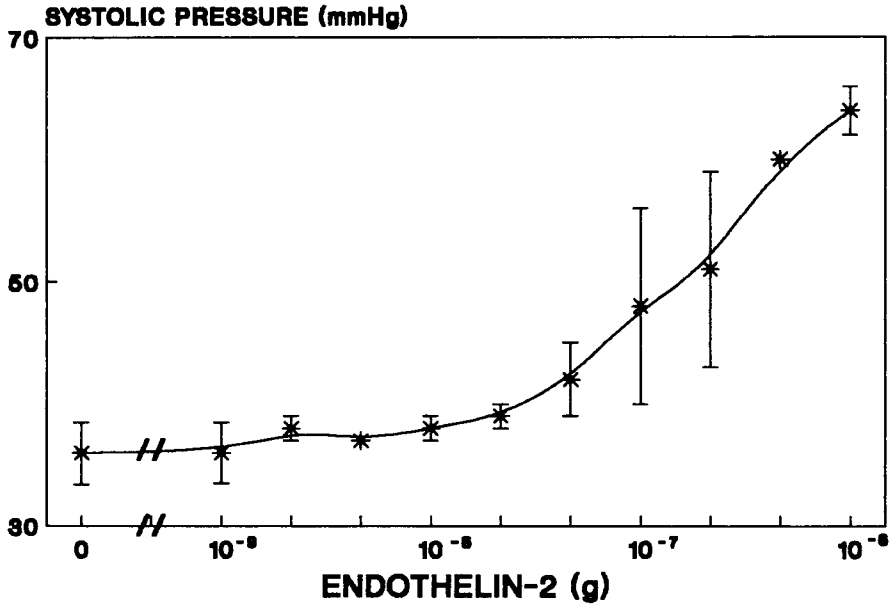

FIGURE 9.3 Dose response curve for endothelin-2 action on maximum systolic ventricular pressure of constant flow-perfused isolated rat hearts. Details as for Figure 9.2. The mean EC_{50} for the positive inotropic response was 185 ng endothelin-2 per heart.

4) Effects of endothelin-2 on mechanical function in control and reperfused hearts.

i) Effect on systolic developed pressure and aortic output: Measurement of systolic developed pressure reflected the effect of endothelin-2 on the inotropic state of the heart. Under constant flow perfusion conditions (Langendorff mode, 1.25 mmol/l Ca^{2+}), the baseline systolic developed pressure was 36 ± 2.3 mmHg, the threshold dose for the positive inotropic effect was $\approx$50 ng, the maximum systolic developed pressure 65 ± 1.8 mmHg (+ 81 % increase), and the EC_{50} was 185 ng per heart (Figure 9.3).

In the isolated working heart, the same dose of endothelin-2 (100 ng) had no significant influence on aortic output (inotropy) over 40 minutes post application (Table 9.4). Coronary flow was reduced, albeit not significantly ($p>0.05$ at all time points).

TABLE 9.4 Time-dependence of aortic output (AO) and coronary flow (CF) in control working hearts (no ischaemia in the absence and presence of 100 ng endothelin-2.

	Control		
	AO (ml/min)	**AO recovery (%)**	**CF (ml/min)**
Reference	45.1±2.4	100	15.6±0.5
10 min	44.7±4.2	99±2.0	15.3±0.4
20 min	44.0±2.2	98±2.0	15.6±0.5
30 min	43.3±2.4	96±1.9	15.6±0.5
40 min	42.7±2.6	95±2.4	15.3±0.4
	+100ng Endothelin		
	AO (ml/min)	**AO recovery (%)**	**CF (ml/min)**
Reference	44.0±2.0	100	16.0±2.0
10 min	46.0±2.4	105±4.5	12.5±1.5
20 min	44.0±4.0	101±3.0	13.5±1.5
30 min	40.5±3.5	93±4.0	14.0±2.0
40 min	39.5±2.0	90±2.0	14.5±1.5

Reference refers to the reference point 10 min after onset of work (=pre-ischaemic control of Table 9.5) followed, in this series, by 20 minutes normoxic perfusion. 10, 20, 30, and 40 minutes refers to the corresponding time points after this 20 minutes normoxic perfusion and thus correspond to the same time points post-ischaemia of Table 9.5 and Figure 9.4. The aortic output recoveries and coronary flow rates in the presence of endothelin are not significantly different from the respective control values ($p>0.05$).

ii) Effect on post-ischaemic reperfusion function: Since endothelin is a positive inotropic agent, the influence of endothelin on heart function during post-ischaemic reperfusion was studied. The influence of a 20 minutes global ischaemic period on reperfusion function in controls and hearts treated with 100 ng or 200 ng endothelin-2 at the onset of reperfusion is shown in Figure 9.4 and Table 9.5 Aortic output was significantly lower between 10 and 40 minutes of reperfusion compared to pre-ischaemic controls ($p<0.01$ and 0.05, respectively).

100 ng endothelin-2 given at the onset of reperfusion improved heart performance up to 20 minutes and worsened it thereafter (20 to 40 minutes). These changes may partly be due to concomitant changes in coronary flow which was reduced time-dependently during reperfusion ($p<0.05$ up to 30 minutes). The higher dose of endothelin (200 ng) reduced aortic output throughout reperfusion compared to the corresponding control values ($p<0.05$, except at 10 minutes). The coronary flow reduction was similar to that observed with the lower dose of endothelin.

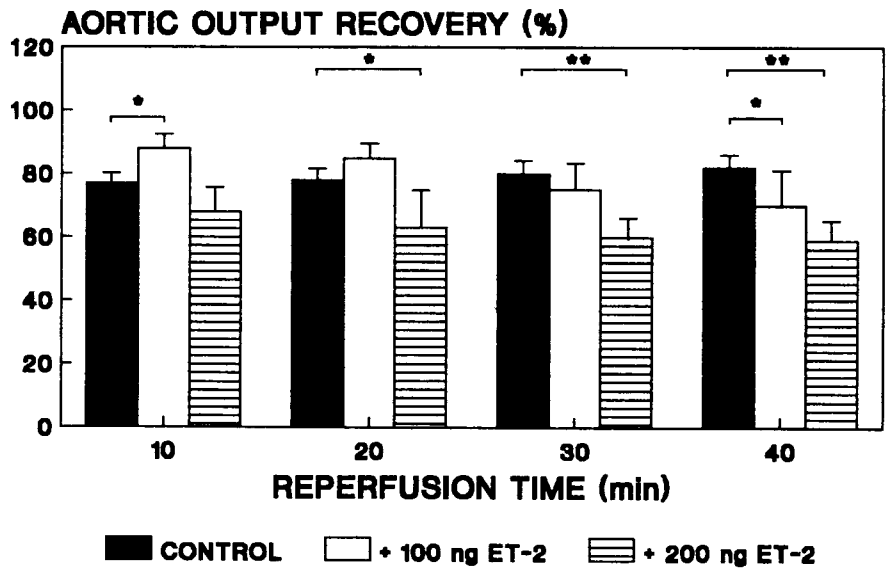

FIGURE 9.4 *Influence of endothelin-2 on recovery of aortic output on reperfusion. The post-ischaemic aortic output recovery between 10 and 40 minutes of reperfusion in the absence (Control) and presence of 100 or 200 ng endothelin-2 given at the onset of reperfusion was plotted. For pre-ischaemic reference values see Table 9.5.*
Means ± S.E.M., n=7 (control and 100ng endothelin-2) and 3 (200 ng endothelin-2). * *$p<0.05$ vs control.*

TABLE 9.5 Influence of endothelin-2 on recovery of aortic output (AO) after ischaemia. Coronary flow (CF) is given for comparison.

Time point	AO (ml/min)	Control AO recovery (%)	CF (ml/min)
Control	48.0±2.2	100	16.3±1.2
10 min	36.6±2.2[1]	77±1.9	16.1±1.1
20 min	37.3±2.1[1]	78±1.9	16.2±1.2
30 min	38.7±2.2[2]	80±1.7	16.6±1.3
40 min	39.8±2.1[2]	82±1.8	16.5±1.3
Time point	**AO (ml/min)**	**+100 ng endothelin AO recovery (%)**	**CF (ml/min)**
Control	47.0±3.4	100	16.8±1.4
10 min	41.1±3.8	88±3.4[4]	11.3±1.1[1]
20 min	39.7±3.2	85±3.3	11.9±1.1[2]
30 min	36.1±3.5[2]	75±4.9	12.7±1.2[2]
40 min	34.5±4.1[2]	70±4.3[4]	13.7±1.2
Time point	**AO (ml/min)**	**+200 ng endothelin AO recovery (%)**	**CF (ml/min)**
Control	44.5±5.5	100	17.0±1.0
10 min	30.0±1.4	68±4.0	13.0±0.6[2]
20 min	28.0±1.4	63±5.5[4]	13.5±0.5[2]
30 min	26.5±2.5	60±1.5[3]	14.5±0.5
40 min	25.5±2.5	59±1.4[3]	15.0±1.0

Values are mean ± SEM, n=7 (100ng ET-2). The experimental protocol is described in the Methods. [1]$p<0.01$ *vs*, pre-ischaemic value; [2]$p<0.05$ *vs.* pre-ischaemic value; [3]$p<0.01$ *vs.* control (no endothelin); [4]$p<0.05$ *vs.* control.

Summary of findings of study. 1) Immunoreactive endothelin was released under basal, ischaemic and reperfusion conditions in picogram amounts.

2) Endothelin release rate was reduced during ischaemia and increased on reperfusion.

3) Exogenous endothelin caused coronary vasoconstriction in reperfused hearts with a 30-fold higher potency than in hearts not subjected to ischaemia.

4) Mechanical reperfusion function was initially improved and later harmed by high doses of exogenous endothelin given at the onset of reperfusion.

CHAPTER 10

DISCUSSION, SUMMARY AND CONCLUSIONS

10.1 Possible Mechanisms Contributing to Reperfusion Ca^{2+} Overload and Stunning.

10.1.1 Sarcolemmal Ca^{2+} Fluxes.

i) Enhanced Ca^{2+} influx at reperfusion. Data obtained in this study (chapter 5) indicate that increased cytosolic Ca^{2+} overload at the onset of reperfusion by compounds that increase transsarcolemmal Ca^{2+} fluxes, exacerbates reperfusion stunning (Figure 5.2).

Although cytosolic Ca^{2+} overload is proposed as one major cause of mechanical stunning (Bolli, 1990), thus far the evidence has been indirect. Marban and co-workers (1989) measured intracellular Ca^{2+} during ischaemia and 2.5 minutes after reperfusion using nuclear magnetic resonance spectrometry. During ischaemia, they observed an increase in time averaged intracellular free Ca^{2+} which had decreased to normal (or below normal) within 2.5 minutes of reperfusion. They did not however measure intracellular free Ca^{2+} levels at the onset of reperfusion. In a recent study Kusuoka and co-workers (1990) found substantially increased systolic Ca^{2+} transients in reperfused stunned hearts, but do not report on findings right at the onset of reperfusion. It therefore remains uncertain whether there is an increase in free cytosolic Ca^{2+} during early reperfusion. In this study (chapter 5), the hypothesis that Ca^{2+} influx (and possible cytosolic Ca^{2+} 'overload') is responsible for post ischaemic stunning was tested as certain interventions at the time of reperfusion or before ischaemia may be expected to reduce or exacerbate reperfusion injury and stunning. The use of Ca^{2+} influx enhancing procedures at the time of reperfusion exacerbated stunning. This finding supports Kusuoka and co-workers (1987) who by using low extracellular Ca^{2+} concentrations in the reperfusion solution improved post-

ischaemic mechanical function. Low Ca^{2+} (0.5 mM) reperfusion solutions were also used by Kuroda and co-workers (1986) with improved reperfusion recoveries, but these experiments were performed with Ca^{2+} concentrations of 2.5 mM in the working phase when mechanical function measurements were made. This makes direct comparisons between the two sets of data difficult. The data obtained during the present study indicate that there is a dose dependent decrease in recovery of function with an increase in Ca^{2+} concentration of the early reperfusion solution.

Similar trends were obtained using the positive inotropic agents Bay K 8644, isoproterenol or forskolin during early reperfusion. They all had a detrimental effect on the post-ischaemic mechanical function when given at the time of reperfusion. Although the effect of a low Ca^{2+} concentration in the reperfusion solution has been investigated before, the use of inotropes at the onset of reperfusion, and their effects on function have not been studied. The effects of inotropes (late in reperfusion) on the already stunned heart has been investigated (Mercier *et al.*, 1982; Ellis *et al.*, 1983; Becker *et al.*, 1986) to ascertain their ability to improve contractile function in these stunned hearts. There appears to be a short period during early reperfusion when the heart is more susceptible to reperfusion damage caused by Ca^{2+} influx and overload. The use of agents enhancing Ca^{2+} influx should therefore be restricted to "late" reperfusion when the heart is less prone to Ca^{2+} induced damage.

ii) Inhibition of Ca^{2+} influx. In contrast, agents thought to inhibit Ca^{2+} fluxes (nisoldipine), when given at the time of reperfusion, improved post-ischaemic mechanical function (Figure 5.3). These agents included nisoldipine, one of the more specific organic Ca^{2+} antagonists (Kass, 1983) and the inorganic Ca^{2+} channel blockers Mn^{2+} or Mg^{2+}. Although magnesium is a normal constituent

of the extracellular fluid, it has Ca^{2+} channel blocking properties when used in high concentrations (Lansman, Hess and Tsien, 1986). Manganese is also a strong Ca^{2+} channel blocking agent but at much lower concentrations (Kass and Tsien, 1975). Both Mn^{2+} and Mg^{2+} maintained mechanical arrest during their infusion in the early reperfusion period. It was not clear whether the protective effect lay in their ability to maintain mechanical arrest during early reperfusion or their Ca^{2+} blocking action. In this study a high K^{+} (21 mM) solution at the time of reperfusion maintained arrest, but did not improve functional recovery of the heart. When a high K^{+} was used in conjunction with Mn^{2+} or the high Mg^{2+} solution, the functional recoveries were significantly improved and were comparable with those of the groups of hearts reperfused with a normal K^{+} concentrations and one of the cations added. Therefore the protection with high Mg^{2+} or Mn^{2+} was not acting by inducing mechanical arrest during the early reperfusion period. Data obtained using these inorganic Ca^{2+} channel blockers lends further support to the hypothesis that the prevention of Ca^{2+} influx at the time of reperfusion has a protective effect on the post-ischaemic heart.

Surprisingly, nisoldipine, when given at the onset of reperfusion, improved reperfusion function in the rat heart model. Calcium antagonists have been used during reperfusion in an attempt to reduce Ca^{2+} influx, but Ca^{2+} influx was merely delayed (rather than prevented) after 30 or 60 minutes of ischaemia (Nayler *et al.*, 1988). This finding may be due to the fairly long ischaemic episode used in that study causing more severe ischaemic and subsequent reperfusion damage. Reperfusion after 30 minutes ischaemia, may cause irreversible injury (Jennings *et al.*, 1985). These authors found that no long term protection was achieved when nifedipine was used in the reperfusion solution after these extended periods of ischaemia (Nayler *et al.*, 1988). Calcium antagonists have previously been used to attenuate stunning (Nayler, Buckley and Leong, 1990;

Przyklenk and Kloner, 1988; Przyklenk *et al.*, 1989). The treatment was commenced before ischaemia and maintained throughout ischaemia and reperfusion (Nayler, Buckley and Leong, 1990), or given only during reperfusion or 30 minutes after reperfusion (Przyklenk and Kloner, 1988). The fact that Ca^{2+} antagonists have marked haemodynamic and vasodilator effects make the interpretation of the latter groups results difficult. It is possible that the beneficial effects seen may be due to increased coronary flows and/or a reduction in afterload (systemic blood pressure) in this model. However, in one specific study, low doses of intracoronary nifedipine were used late in reperfusion to attenuate stunning in the absence of any systemic haemodynamic changes (Przyklenk *et al.*, 1989). The mode of action of the late administration of this drug is unknown but may relate to the presence of formed blood cells present in the dog model and absent from our isolated crystalloid perfused rat heart model.

Based on these findings, we have proposed a *two-phased model for Ca^{2+} as pathogen for stunning* (Opie and Du Toit, 1992). *In the first and causative phase, there may be a brief period of cytosolic Ca^{2+} overload which is followed by the second phase associated with hypocontractility and an apparent shortage of activator Ca^{2+}.*

10.1.2 Possible Role for the Sarcoplasmic Reticulum in Ischaemia/Reperfusion Cytosolic Ca^{2+} Overload and Stunning. The role of the SR in mechanical stunning has thus far been explored with limited success. Besides the entry of Ca^{2+} by the voltage dependent Ca^{2+} channels, another possible source of cytosolic Ca^{2+} overload is the sarcoplasmic reticulum (SR) (Pesaturo and Gwathmey, 1990). Reperfusion with sudden generation of free radicals (Hearse, Humphrey and Bullock, 1978) may be associated with release of Ca^{2+} from the

SR (Shattock, Hearse and Matsuura, 1990), so that interventions that reduce such Ca^{2+} release and recycling may attenuate stunning.

i) Reperfusion function following sarcoplasmic reticulum Ca^{2+} release inhibition during ischaemia and/or reperfusion. Ryanodine is thought to be an agent which in low concentrations acts to open the Ca^{2+} channel of the SR (Rousseau, Smith and Meisner, 1987), whereas higher concentrations close the channel (Marban and Wier, 1985; Inui *et al.*, 1988) thus effectively acting as an internal Ca^{2+} blocker which inhibits intracellular Ca^{2+} release and oscillations (Kihara and Morgan, 1991). In this study, the use of ryanodine before ischaemia or at the onset of reperfusion, improved reperfusion function (Figure 5.3). The ryanodine concentration we used was low ($3x10^{-9}$ M) but was similar to that having a negative inotropic effect in the perfused rat heart (Thandroyen *et al.*, 1988), and close to that (10^{-8} M) which decreased the Ca^{2+} signal and peak systolic pressure in the isolated rat atria (Northover, 1991). Infusion of ryanodine at the time of reperfusion attenuated stunning as shown by a better post-ischaemic mechanical function. These data support a role for excess Ca^{2+} cycling through the SR or release of Ca^{2+} from the SR in the genesis of stunning.

Possible mechanism of protection of ryanodine: Kusuoka and co-workers (1990) reported that the stunned heart exhibited an increase in amplitude of Ca^{2+} transients (diastolic/systolic). They suggest that these increases in cytosolic Ca^{2+} transients would require that more ATP be spent in Ca^{2+} sequestration which may in turn explain the decreased efficiency of energy utilization of the stunned myocardium (Laster *et al.*, 1989). This proposal may also explain why some research groups (Krause, Jacobus and Becker, 1989; Limbruno *et al.*, 1989) found decreased function of the SR Ca^{2+} ATP-ase pump in the stunned heart.

Inadequate tissue ATP in direct proximity to the SR may cause decreased pump function if ATP were compartmentalised in the myocyte (Bricknell, Daries and Opie, 1981). Ryanodine by its Ca^{2+} modulating action may hypothetically decrease excessive Ca^{2+} transients, thus helping to remove the cause of stunning.

ii) Reperfusion function following SR Ca^{2+} ATP-ase pump inhibition during ischaemia and/or reperfusion.

Ryanodine (chapter 5) and ruthenium red (Benzi and Lerch, 1992), two inhibitors of release of Ca^{2+} from the SR when given during reperfusion, are both able to improve reperfusion stunning, thereby supporting the concept that excess cycling of Ca^{2+} may contribute to post-ischaemic mechanical dysfunction. Because it is believed that ischaemic and reperfusion induced cytosolic Ca^{2+} overload (Bolli, 1990), or excess reperfusion intracellular Ca^{2+} cycling (Kusuoka *et al.*, 1990; Benzi and Lerch, 1992) may exacerbate stunning, we tested the effects of Ca^{2+} ATP-ase pump inhibitors given before ischaemia and during reperfusion mechanical function (stunning). Given before ischaemia, these ATP-ase pump inhibitors may cause SR and cytosolic Ca^{2+} depletion by increased Na^{+}/Ca^{2+} exchange activation (Baudet, Shaoulian and Bers, 1993) and given during reperfusion, these compounds may decrease potentially harmful Ca^{2+} oscillations and ATP wastage.

Pre-treatment of the isolated rat heart with either cyclopiazonic acid or thapsigargin in the appropriate concentrations before ischaemia attenuated reperfusion stunning in this study (chapter 7). These improved functional recoveries may have been due to drug induced depletion of SR Ca^{2+} which could attenuate the extent of Ca^{2+} overload that occurs during ischaemia and reperfusion, because inhibition of the Ca^{2+} uptake pump is associated with i)

depletion of Ca^{2+} from the SR, and ii) increased activity of the sarcolemmal Na^{+}/Ca^{2+} exchanger to extrude Ca^{2+} (Baudet, Shaoulian and Bers, 1993). It is of interest that Mitchell and co-workers (1992) found that pre-ischaemic depletion of SR Ca^{2+} using thapsigargin or ryanodine could mimic preconditioning in the isolated rat heart model. Their proposed mechanism of pre-conditioning is that inositol triphoshate induces Ca^{2+} release from internal Ca^{2+} stores (SR) before ischaemia, decreases the intracellular Ca^{2+} levels in these stores and thus decreases intracellular Ca^{2+} overload during ischaemia and reperfusion. Similarly, high Mg^{2+} containing cardioplegic arrest solutions, which are thought to deplete the SR of Ca^{2+}, attenuate the severity of ischaemic injury when compared with high K^{+} arrest solutions which are thought to induce pre-arrest SR Ca^{2+} loading (Steenbergen *et al.*, 1990).

Possible mechanism of protection of CPA and thapsigargin: If one assumes that the elevated cytosolic Ca^{2+} levels associated with ischaemia require that more Ca^{2+} be sequestered into the SR and more ATP be consumed (Barry *et al.*, 1987; Benzi and Lerch, 1992), transient inhibition of the Ca^{2+} ATP-ase pump activity would be expected to have an ATP sparing effect in ischaemic tissue.

Although it has been proposed that increased Ca^{2+} cycling during ischaemia may cause elevated ATP consumption (and wastage) (Barry *et al.*, 1987), the data obtained in this study (chapter 7) indicate that CPA and thapsigargin did not have an ATP sparing effect on the ischaemic heart. Post-ischaemic ATP levels of thapsigargin (10^{-6} and $2.5x10^{-8}$ M) treated hearts were lower than those of control and CPA treated hearts. Despite similar tissue ATP levels at the end of ischaemia, there was a difference in functional recoveries observed when comparing the two thapsigargin treated groups with one another. This finding

would suggest that the protection afforded by these ATP-ase pump inhibitors is not related to high energy phosphate preservation. Although thapsigargin (10^{-6} and $2.5X10^{-8}M$) decreased tissue ATP levels, reperfusion function with the low concentration ($2.5X10^{-8}M$) of the compound was better than with the higher concentration.

Specificity of these compounds: Cyclopiazonic acid is a specific and very potent inhibitor of the SR Ca^{2+} ATP-ase pump of cardiac (Lahouratate, Quiniou and Leoty, 1992; Mitchell *et al.*, 1992; Pery-man *et al.*, 1993), and vascular smooth muscle (Shima and Blaustein, 1992). Although no data are available to quantify the percentage inhibition of cardiac SR Ca^{2+} ATP-ase pumps by cyclopiazonic acid, it totally inhibits caffeine-evoked increases in tension in vascular smooth muscle (Shima and Blaustein, 1992) and Ca^{2+} transport into isolated canine cardiac SR vesicles (Lahouratate, Quiniou and Leoty, 1992).

It has recently been shown that both cyclopiazonic acid and thapsigargin are able to partially block SR Ca^{2+} uptake in multicellular rabbit cardiac preparation (Baudet, Shaoulian and Bers, 1993). These authors (Baudet, Shaoulian and Bers, 1993) also found that although both compounds decreased Ca^{2+} uptake into the SR, cyclopiazonic acid had the more expected effect in that it significantly slowed relaxation time in their preparation. This was in contrast with thapsigargin that slowed contraction but had very little effect on myocyte relaxation time. From these data, and our own findings, it would seem that thapsigargin may have other sites of action in the intact myocyte which may explain the unexpected data we obtained with high concentrations of the compound.

Concentration related effect of thapsigargin: A low concentration of thapsigargin given at the onset of reperfusion was ineffective in preventing stunning. This finding may be due to only partial inhibition of Ca^{2+} ATP-ase pump (Thastrup *et al.*, 1990; Shima and Blaustein, 1992) which would allow continued Ca^{2+} cycling between the SR and the cytosol. Although a higher concentration of thapsigargin (10^{-6}M) only had a weak negative inotropic action in the non-ischaemic heart, it worsened reperfusion stunning when given before ischaemia or at the onset of reperfusion. The detrimental effects of high concentrations of the compound may be ascribed to its ability to increase cytosolic Ca^{2+} concentrations by causing Ca^{2+} leakage from unidentified Ca^{2+} stores (Thastrup *et al.*, 1989). The effect of high concentrations of thapsigargin (given during reperfusion) on reperfusion function mimic those observed with inotropes in other parts of this study (chapters 5 & 9). During reperfusion, there was an initial increase, followed by a later decrease in mechanical function.

Ischaemic contracture was used as an index of cytosolic Ca^{2+} levels during ischaemia. Although there were no differences in the time to onset of contracture, the amplitude of ischaemic contracture was higher in hearts treated with the high thapsigargin concentration than in the CPA and low thapsigargin treated hearts. This may indicate that the higher concentrations of thapsigargin increased the ischaemic Ca^{2+} concentrations. The transient increase in the cytosolic Ca^{2+} levels observed in vascular smooth muscle (Dick and Sturek, 1993) during thapsigargin (10^{-6}M) treatment, may also occur in myocytes, and could explain the increase in the magnitude of ischaemic contracture (a reflection of Ca^{2+} levels) observed after pretreatment with this concentration of thapsigargin.

Reservations of studies with thapsigargin and cyclopiazonic acid: Although these compounds protected against stunning, they are both negatively inotropic agents when used in the working heart model (chapter 7). Data concerning the inotropic action of thapsigargin in cardiac tissue are at present scarce and contradictory. Data presented in chapter 7 show that thapsigargin is a weak negative inotrope in the working heart model but not in the Langendorff model which is in accord with findings in isolated guinea pig myocytes (Lewartowski and Wolska, 1993). These data are contradicted by the finding that thapsigargin almost abolishes cell shortening in isolated rat ventricular myocytes (Kirby *et al.*, 1992) at virtually the same concentration.

Another weakness is that our study and those of others cannot fully exclude other sites of action for the two compounds studied. Nonetheless, these compounds appear to be valuable tools to aid the study of intracellular Ca^{2+} fluxes in cardiac tissue. Extensive further clinically applicable investigation would however be needed before any possible application to patients.

iii) The possible role of the sarcoplasmic reticulum in mechanical stunning. Two independent studies (Limbruno *et al.*, 1989; Krause, Jacobus and Becker, 1989) examined the function of the Ca^{2+} ATP-ase pump of SR vesicles obtained from stunned hearts. They found that ATP-ase pump function of these vesicles was less effective in sequestering Ca^{2+} than the ATP-ase pumps from normal hearts. Other than the Ca^{2+} uptake abnormalities, some studies have implicated the release channels of the SR in stunning (Hohl, Galeb and Altschuld, 1992; Davis, Lebolt and Feher, 1992).

Contrary to these findings, Kusuoka and co-workers (1990) found that intracellular Ca^{2+} transients were augmented in the stunned reperfused heart.

Their findings were supported by those showing that Ca^{2+} uptake of stunned SR Ca^{2+} ATP-ase vesicle was 17% higher than that of control vesicles (Lamers *et al.*, 1993). These increased Ca^{2+} oscillations should be associated with increased SR Ca^{2+} ATP-ase pump activity, which may explain the increased oxygen consumption of the stunned myocardium (Laster *et al.*, 1989). These data would imply that neither release from, nor uptake of Ca^{2+} into the SR is at fault, but that the contractile apparatus in the stunned heart may be less sensitive to activator Ca^{2+} during early reperfusion.

10.1.3 Role of the Na^+/H^+ Exchanger in Stunning.

In this study (chapter 6), inhibition of Na^+/H^+ exchange activity before ischaemia or at the onset of reperfusion attenuated reperfusion stunning (Figure 6.1 and Figure 6.5). Although a transient increase in cytosolic Ca^{2+} levels associated with stunning has been linked to Ca^{2+} entry via the Ca^{2+} channel (Chapter 5; Opie and Du Toit, 1992), other entry pathways may contribute to this Ca^{2+} overload. The possible contribution of the Na^+/H^+ exchanger has been explored but with limited success mainly due to the nonspecificity of the inhibitors previously available. Also, these compounds when given throughout the experiment (Karmazyn, 1988; Sack *et al.*, 1992) or before ischaemia (Scholz *et al.*, 1992; Tani and Neely, 1989; Murphy *et al.*, 1991), may by an anti-ischaemic action indirectly decrease reperfusion stunning.

The myocardial Na^+/H^+ exchanger is stimulated by a decrease in intracellular pH or an increase in extracellular pH (Lazdunski, Frelin and Vigne, 1985). During ischaemia, myocyte pH decreases (Jacobus *et al*,. 1977) and Na^+ increases (Regan *et al*,. 1980; Jennings *et al*,. 1985; Tani and Neely, 1989; Murphy *et al*,. 1991). Lazdunski and co-workers (1985) hypothesised that the low intracellular pH during ischaemia would activate the Na^+/H^+ exchanger on

reperfusion, thereby increasing Na^+ accumulation during early reperfusion. This proposal was confirmed by Tani and Neely (1989) who showed that there was a slow gradual increase in tissue Na^+ levels during 30 minutes ischaemia followed by a sudden sharp rise during reperfusion. Amiloride pretreatment largely eliminated this rapid Na^+ rise during reperfusion, but had very little effect on the slow influx of Na^+ during ischaemia. During ischaemia, Na^+ may thus enter the myocyte though the previously described (Ver Donck and Borgers, 1991) modified Na^+-channel. Tani and Neely (1989) also found that the intracellular Na^+ level after 2 minutes reperfusion was linearly related to the $^{45}Ca^{2+}$ uptake and to depression of reperfusion function. Reperfusion of the ischaemic heart with an acidotic solution (Kitikaze, Weisfeldt and Marban, 1988) also decreases reperfusion stunning. The improved mechanical function is presumably due to reduced Na^+/H^+ and consequently less Na^+/Ca^{2+} exchanger activity during reperfusion.

Tani and Neely (1989) who studied a high concentration of amiloride ($10^{-3}M$) given upon reperfusion only, used a relatively short ischaemic time of 15 minutes in one protocol and 30 minutes in another protocol. After the short ischaemic time they could show no evidence of mechanical stunning in the Langendorff model. On the other hand, after 30 minutes ischaemia the very low mechanical function during reperfusion and the high uptakes of cytosolic $^{45}Ca^{2+}$ may suggest that some of the myocardium was irreversibly injured, so that by definition (see details of definition in chapter 4), true stunning was not present. Thus there has as yet been no direct evidence favouring the view that inhibition of Na^+/H^+ exchange specifically during the reperfusion period could prevent reperfusion stunning and arrhythmias.

Amiloride specificity: The data obtained here and elsewhere with amiloride suffer from the defect that it is a relatively non-specific compound. Besides inhibiting Na^+/H^+ exchange, amiloride also inhibits i) the Na,K-ATP-ase (Kennedy *et al.*, 1986), ii) the Na^+/Ca^{2+} exchanger in myocardial tissue (Kennedy *et al.*, 1986), iii) sodium channels in various tissues (data not available for cardiac tissue) (Benos, 1982) and iv) sarcolemmal T-type Ca^{2+} channels (Tang, Presser and Morad, 1988). Protection by amiloride against reperfusion injury may thus be afforded by any one, or, all of these properties of the compound. In studies where the inhibitor was given before ischaemia and/or during ischaemia (Scholz *et al.*, 1992; Tani and Neely, 1989; Dennis *et al.*, 1990; Karmazyn, 1988; Sack *et al.*, 1992), the compound may have had an anti-ischaemic action. If the Na^+ channel blocker lidocaine has anti-ischaemic properties (Gerstenblith *et al.*, 1978; Wilson *et al.*, 1993), then it would be reasonable to assume that any blocking properties that amiloride may have on the cardiac Na^+ channel, would also be anti-ischaemic. This possible anti-ischaemic property of the compound would also be expected to decrease reperfusion injury (Opie, 1989b).

Possible reasons for discrepancies between data obtained using the Langendorff vs working heart model: Amiloride was used as a reference compound to compare its effects with those of HOE 694. We used an amiloride concentration (10^{-3}M) which protected the Langendorff perfused rat heart model against ischaemic and reperfusion damage after 30 minutes ischaemia (Tani and Neely, 1989). In our study, this concentration protected the Langendorff (non-working) preparation (Figure 6.8), but not the working heart model against reperfusion stunning (Figure 6.3). The working heart model was, however, protected against reperfusion stunning by lower concentrations (10^{-5}M) of the compound. The exact reason for this discrepancy in the functional

recovery between the two models is not clear but may be explained by one, or both, of the following factors. First, the Langendorff perfused (left ventricular balloon containing) heart performs isovolumic contractile work while the working heart performs both pressure and volume work. Hence, it may be possible that the higher concentrations of amiloride together with ischaemia, detrimentally affected the recovery of the working heart. These differences may therefore be due to the different work loads to which the types of perfused hearts are exposed. Second, judging by the very good (when compared with the working heart model) functional recoveries recorded with the Langendorff model after 30 minutes ischaemia (our observation and those of Tani and Neely (1989)), it seems that the Langendorff model is not as sensitive a measure of functional recovery as the working heart model. The isovolumic Langendorff model may not be as critical in measuring and accentuating diastolic (muscle relaxation) abnormalities in the stunned heart. If diastolic abnormalities are an important facet of stunning (which they appear to be), then these abnormalities may not be detected in the isovolumic model. The working heart model relies on normal relaxation characteristics for proper left ventricular filling to take place. Slower diastolic relaxation would decrease the diastolic left ventricular filling time and consequently decrease aortic output values. This matter needs further investigation to clarify the discrepancies observed.

Reservations of these studies performed with HOE 694: Although HOE 694 is thought to be a specific and potent inhibitor of the Na^+/H^+ exchanger, few publications are available. Schmid and co-workers (1992) have shown that HOE 694 is a potent inhibitor of the Na^+/H^+ exchanger in porcine cerebral capillaries. Data concerning its specificity on cardiac Na^+/H^+ exchange are not available. The absence of a dose-dependent effect with the three concentrations of HOE 694 tested in this study (chapter 6), may indicate that too narrow a

concentration range was used. It may be that the three concentrations used were all high enough to cause near-total inhibition of the Na^+/H^+ exchanger in this model.

10.1.4 Role of Na^+/Ca^{2+} Exchanger in Stunning.

Increased intracellular Na^+ levels should activate the Na^+/Ca^{2+} exchanger and lead to Ca^{2+} influx. Grinwald and Bosnahan (1987) have shown that elevated intracellular Na^+ levels during hypoxia could indirectly contribute to Ca^{2+} overload in the reoxygenation period. In support of these findings, Murphy and co-workers (1991), examined ischaemic myocardial Na^+ and Ca^{2+} gain using nuclear magnetic resonance. They showed that amiloride pretreatment decreased the intracellular rise of these two ions during ischaemia and improved reperfusion mechanical function. Reperfusion with a high extracellular Na^+ solution (Kusuoka, De Hurtado and Marban, 1993) decreases reperfusion stunning, presumably by restricting reperfusion Na^+ efflux and Ca^{2+} influx via the Na^+/Ca^{2+} exchanger. The data presented by these groups (Murphy *et al.*, 1991; Grinwald and Bosnahan, 1987) suggests that the Na^+/Ca^{2+} exchanger contributes to transient reperfusion cytosolic Ca^{2+} overload which could predispose to reperfusion stunning according to the proposals made earlier in this thesis (chapter 5).

Mechanism of Ca^{2+} induced stunning: Early cytosolic Ca^{2+} overload has been proposed as a mechanism for reperfusion arrhythmias, acting by induction of the transient inward current, (I_{ti}) (Opie and Coetzee, 1988). Furthermore net Ca^{2+} overload is thought to be instrumental in the genesis of other aspects of reperfusion injury, such as accelerated cell necrosis (Kass and Tsien, 1975). The actual mechanism of the Ca^{2+} induced damage to the contractile mechanisms is still obscure. The data obtained with Mn^{2+} and Mg^{2+} added to the K^+-arrested

hearts show that inhibition of contractile activity of the heart during early reperfusion cannot be the mechanism. Rather, excess Ca^{2+} entry by the voltage dependent Ca^{2+} channel and indirectly, by Na^{+}/H^{+} exchange activation appeared to play a role. Also, the SR may act as a Ca^{2+} source or deplete cytosolic ATP levels by perpetuating cytosolic Ca^{2+} oscillations. Speculating, excess cytosolic Ca^{2+} could have activated Ca^{2+} dependent enzymes proteases and phospholipases, with consequent damage to contractile organelles (Kass and Tsien, 1975), which would account for the proposed decrease in the responsiveness of the myofilaments to Ca^{2+} (Marban, 1991). Another possibility is that the Ca^{2+} antagonist could directly interact with the cell membrane to prevent the formation of free-radicals (Koller and Bergmann, 1989; Weglicki, Mak and Simic, 1990), however this proposal would appear to be excluded by the data with the Mn^{2+} and Mg^{2+}. A remaining possibility is that excess cytosolic Ca^{2+} is able to enhance formation of free-radicals (McCord, 1985).

The use of Ca^{2+} antagonists late in reperfusion in our isolated non-blood perfused heart did not improve mechanical function (Figure 5.5). The phenomenon of improved function with Ca^{2+} antagonist treatment late in reperfusion (Przyklenk *et al.*, 1989) may be specific to the blood perfused heart and may thus not be relevant in the isolated rat heart model.

10.2 Abnormal Cytosolic Ca^{2+} Fluxes and Arrhythmias.

10.2.1 Role for the Ca^{2+} Channel in Ischaemic and Reperfusion Arrhythmias.

Calcium influx via sarcolemmal Ca^{2+} channels may contribute to the elevated cytosolic Ca^{2+} levels seen during ischaemic and reperfusion ventricular fibrillation. There is sound experimental evidence that Ca^{2+} antagonists pretreatment can prevent ventricular arrhythmias during myocardial ischaemia and reperfusion (Thandroyen, 1982; Lubbe, McLean and Nguyen, 1983; Clusin *et*

al., 1982; Clusin *et al.*, 1984). The heterogeneity of the action of Ca^{2+} antagonists however make it difficult to determine whether these compounds have their anti-arrhythmic action by decreasing the Ca^{2+} current or by their possible anti-ischaemic action. The efficacy of these channel blockers in decreasing reperfusion arrhythmias are less clear. While these compounds were found to be effective in decreasing reperfusion arrhythmias in the rat heart (Lubbe, McLean and Nguyen, 1983; Weishaar and Bing, 1980), these effects appear to be species related. Some research groups have shown Ca^{2+} antagonists to be antiarrhythmic in the canine model (Coker and Parratt, 1983), while others found these channel blockers to be ineffective (Naito *et al.*, 1981 ; Sheehan and Epstein, 1982). The efficacy of Ca^{2+} antagonist treatment in the prevention of arrhythmias therefore seems species dependent.

10.2.2 Intracellular Ca^{2+} Oscillations and Ventricular Arrhythmias.

Both cyclopiazonic acid and thapsigargin decreased the incidence of ischaemic and reperfusion arrhythmias in this study (chapter 8). Although the exact mechanisms responsible for the initiation of ischaemic and reperfusion arrhythmias are poorly understood, there is good evidence that excess cytosolic Ca^{2+} (Thandroyen, 1982; Clusin, Buchbinder and Harrison, 1983; Ferrier, Moffat and Lukas, 1985; Coetzee and Opie, 1987; Opie *et al.*, 1988; Opie and Coetzee, 1988) and abnormal intracellular Ca^{2+} oscillations (Thandroyen *et al.*, 1988; Koretsune and Marban, 1989) may play a role in the genesis of certain types of ischaemic and reperfusion arrhythmias. These intracellular Ca^{2+} oscillations are thought to be caused by the uptake and release of Ca^{2+} from the sarcoplasmic reticulum overloaded with Ca^{2+} (Kass *et al.*, 1978). Direct intracellular Ca^{2+} measurements show that during electrically induced ventricular fibrillation, intracellular Ca^{2+} levels are elevated (Koretsune and Marban, 1989), and that small physiologic intracellular Ca^{2+} oscillations are

augmented by the transition to ventricular fibrillation (Kihara and Morgan, 1991). Ryanodine, and blocked the augmented Ca^{2+} oscillations associated with ventricular fibrillation (Thandroyen *et al.*, 1988; Kihara and Morgan, 1991).

The incidence of reperfusion arrhythmias is related to the duration and the severity of ischaemia (Manning and Hearse, 1984). The SR Ca^{2+} ATP-ase pump inhibitors (CPA and thapsigargin) tested in chapter 8 were used at a series of reperfusion times to confirm that the anti-arrhythmic properties of these inhibitors were not due to an anti-ischaemic action. These compounds did not merely move the bell-shaped curve to the right, thus delaying the vulnerable period, but decreased the incidence of arrhythmias in absolute terms.

Reservations of these studies: Although these compounds have antiarrhythmic properties, they are negatively inotropic agents when used in the working heart model. The model for the arrhythmia study (chapter 8) was an isovolumic Langendorff preparation, not performing external mechanical work in the conventional sense. Under such conditions, cyclopiazonic acid (CPA) is a negative lusitrope but not a negative inotrope (Pery-man *et al.*, 1993). Our mechanical data in the Langendorff model confirm reports showing that sarcoplasmic reticulum Ca^{2+} ATP-ase pump inhibition with CPA in various models had only a slight negative effect on contractile parameters in the rat (Pery-man *et al.*, 1993) and rabbit papillary (Lahouratate, Quiniou and Leoty, 1992) and neonatal ventricular muscle (Agata, Tanaka and Shigenobu, 1993) preparation. This negative lusitropic effect was only apparent in models using isometric contraction as a measure of function (Pery-man *et al.*, 1993). Hence the improved mechanical function in response to both agents found in the arrhythmia study (Figure 8.8) on the coronary ligated heart cannot be extrapolated to other more physiologic models.

10.2.3 Na^+/H^+ Exchange and Reperfusion Arrhythmias.

The antiarrhythmic properties of high concentrations of amiloride have already been demonstrated in post-ischaemic (Dennis *et al.*, 1990; Karmazyn, 1988) and ouabain-treated hearts (Lotan *et al.*, 1992). We found that HOE 694 was antiarrhythmic at all concentrations used in this study (Figure 6.2 and Table 6.1), and also when only added upon reperfusion (Table 6.2). Our findings suggest that elevated Na^+/H^+ exchange activity plays a role in the genesis of reperfusion arrhythmias. The antiarrhythmic effect of HOE 694 when used in the reperfusion phase only, indirectly supports the hypothesis that a transient cytosolic Ca^{2+} overload explains some reperfusion arrhythmias (Opie and Coetzee, 1988).

10.3 Endothelin and its Possible Involvement in Ischaemic and Reperfusion Injury.

Endothelin release under different experimental conditions.

In the isolated retrogradely perfused rat heart, there were low but constant levels of endothelin released into the coronary effluent under basal conditions. This stability is a prerequisite for the detection of possible changes induced by variations of the experimental protocol, and is consistent with available evidence suggesting that the peptide is continuously released from endothelial cells after intracellular processing (Hexum *et al.*, 1990; Lerman *et al.*, 1990).

In our low-flow model of ischaemia, endothelin release was greatly reduced during the ischaemic period of 90 or 180 minutes. Since the whole effluent sample was collected and processed, loss of endothelin could be ruled out. The low ischaemic flow rate could, however, explain a reduced rate of washout despite the same rate of release, resulting in a reduced rate of appearance in the coronary effluent. Alternatively, the peptide could also be internalized. A

reduced endothelin release rate under low oxygen tension has also been reported for cultured porcine cerebral endothelial cells (Yoshimoto *et al.*, 1991), while hypoxia increased endothelin secretion of cultured human endothelial cells (Kourembanas *et al.*, 1991), mesenteric resistance vessels of the rat (Rakugi *et al.*, 1990) and plasma immunoreactive concentrations in intact anaesthetised rats (Horio *et al.*, 1991). The differences in absolute oxygen and/or carbon dioxide tensions in these models may account for the discrepant findings.

On reperfusion, endothelin secretion was increased in the present study over the basal release rate, albeit statistically significant only in the group which had been subjected to ischaemia for three hours and in the presence of foetal calf serum. Serum of several species has been shown to stimulate endothelin synthesis of endothelial and other cells. When bovine adrenocortical capillary endothelial cells were cultured in the combined presence of Dulbecco's modified Eagle media and bovine serum albumin or foetal calf serum, the spontaneous release of endothelin was two to three times higher than without serum (Hexum *et al.*, 1990). Human serum stimulated the production of endothelin-1 by human vascular endothelial cells (Ristimäki *et al.*, 1991). The enhanced secretion could be dependent on peptides found in the serum, of which at least thrombin, arginine-vasopressin and angiotensin II can stimulate endothelin secretion of cultured cells (Yanagisawa *et al.*, 1988; Emori *et al.*, 1989). More than 90 minutes of ischaemia were necessary in the present model to substantially increase endothelin release. A lack of storage of the preformed peptide and the dependence of secretion on transcriptional control may account for this finding (Hexum *et al.*, 1990). A similar time-dependence of stimulated endothelin release has been reported for other models (Boulanger and Luscher, 1990; Ohlstein *et al.*, 1991).

The present results obtained with a low-flow model of ischaemia are partly at odds with those recently reported by Watanabe and co-workers (1991). They induced regional ischaemia by ligating the left anterior descending coronary artery in intact animals. In their rats, plasma levels were significantly increased both during the reperfusion and occlusion (ischaemic) phase and remained elevated for at least 3 hours after reperfusion. These differences may be due to the totally different myocardial blood flow profiles in models of global low-flow and regional ischaemia, respectively. Additional extracardiac sources of endothelin are likely to have contributed to the increased plasma levels in their study, since the actual (cardiac) tissue levels, albeit also increased during ischaemia and on reperfusion, can only account for a small fraction of this increase (less than 10 %, based on a plasma volume at least 10-times higher than heart weight).

Endogenous vs exogenous endothelin.

The radioimmunoassay used in the present study did not distinguish between the various endothelin isomers, hence the relative amounts of endothelin-1, -2 and -3 in the effluents are not known. Although the majority of published reports deal with endothelin-1, endothelin-2 is a stronger vasoconstrictor and has an even longer duration of action than endothelin-1 (Vane, 1991). We therefore chose this isomer to assess the potential effects of endogenous endothelin on the coronary circulation and myocardium, although the heart itself may not be the site of production of endothelin-2, in order to obtain maximum smooth muscle and long lasting myocyte effects.

Endothelin and ischaemic heart disease.

Based on increased plasma endothelin levels in patients with ischaemic heart disease (Yasuda *et al.*, 1989; Stewart *et al.*, 1991), a possible association of the endothelins with cardiovascular disease has been proposed. Neubauer and co-workers (1990, 1991) have recently shown in an isolated rat heart model that endothelin-1 is a potent coronary constrictor under hypoxic, reoxygenated and reperfusion conditions. Coronary flow was marginally increased (vasodilation) at one dose (40 pmol, $\approx$100 ng) of endothelin-1 in hearts subjected to mild hypoxia, and decreased (vasoconstriction) in hearts subjected to severe hypoxia for 60 minutes. The highest dose (400 pmol, $\approx$1 μg), produced almost complete coronary occlusion irrespective of the model used. These data suggest that, in their model, irreversible injury was necessary for the coronary vasculature to become more sensitive to the constrictor effects of endothelin-1. In another study also using the Langendorff perfused heart at high (35 ml/min) constant coronary flow rates, Stewart and Baffour (1990) found a dose-dependent increase in coronary perfusion pressure and decrease in left ventricular developed pressure in ischaemic hearts. Since endothelium-dependent-relaxing-factor (EDRF)-mediated relaxation was found to be unimpaired, their finding would suggest that either ischaemia could stimulate production of endogenous endothelin by the coronary bed or sensitise the coronary vasculature to the constrictive effects of the exogenously applied peptide.

The present results (chapter 9) confirm previous findings obtained in intact animals and isolated vessel preparations in that endothelin constricted the coronary vascular bed in a dose-dependent manner, resulting in a twofold increase in coronary perfusion pressure within the dose range of 1 ng to 1 μg ($\approx$0.4 to 400 pmol) endothelin-2. On the other hand, exogenous endothelin applied to reperfused hearts subsequent to one hour of low-flow ischaemia was

approximately 30 times more potent a vasoconstrictor than in hearts not previously made ischaemic. This finding supports the proposition that ischaemia and/or reperfusion sensitises the coronary vasculature for endothelin-induced vasoconstriction. Although we have used exactly the same dose range (0.1 to 1000 ng per heart, 0.04 to 400 pmol), it is difficult to compare our findings with those of Neubauer and co-workers (1991), since 1) the degree of ischaemic injury in their models is likely to differ from that in our low-flow model, 2) they used perfusion at constant pressure with measurement of coronary flow, and 3) their dose response curves were not analyzed in terms of EC_{50} values. Nonetheless, our results presented here are in qualitative agreement with theirs.

The total amount of endogenous endothelin released within 30 minutes of reperfusion is about 10 to 30 pg, depending on the duration of ischaemia and the presence or absence of foetal calf serum (Table 9.2). These amounts come close to the threshold doses of exogenous endothelin which elicited an increase of the coronary perfusion pressure in hearts made ischaemic for 1 hour, but not normoxic hearts (Figure 9.2).

Influence of endothelin on recovery of reperfusion mechanical function (myocardial stunning).

In an earlier study (chapter 5) using the working rat heart model, post-ischaemic contractile function is reduced by about 25 % in controls but by about 50 % in hearts subjected to positive inotropic agents given at the onset of reperfusion. Isoproterenol, forskolin, and Bay K 8644 administered during early reperfusion had a detrimental effect on subsequent post-ischaemic mechanical function (stunning). The mechanism involved may be due to early cytosolic Ca^{2+} overloading (Nayler, 1983; Opie, 1989a). However, positive inotropic agents given later in reperfusion to already stunned hearts improved contractile

function (Mercier *et al.*, 1982; Taegtmeyer *et al.*, 1985; Becker *et al.*, 1986). It was therefore of interest to test whether endothelin could contribute to the aetiology of early (up to 10 minutes) or late reperfusion stunning (Opie, 1991c).

To evaluate the effect of endothelin on reperfusion function, its positive inotropic effect had to first be quantified in this model. Surprisingly, the dose of endothelin (100 ng) which produced a consistent, albeit small positive inotropic effect in the Langendorff-perfused heart (Figure 9.3), had no significant influence on aortic output in the working heart compared to controls (Table 9.4). This finding may partly be due to the concomitant vasoconstriction resulting in a reduced coronary flow (Table 9.5) which was avoided in the Langendorff model by perfusion at a constant flow rate. Hence, the potential positive inotropic effect of 100 ng endothelin on post-ischaemic reperfusion work is largely offset by coronary vasoconstriction.

Ideally, the influence of endothelin on reperfusion function should be assessed at several endothelin concentrations. We chose two large doses greatly exceeding the amounts released on reperfusion in the present model (Table 9.2) and which, based on published evidence (Vigne *et al.*, 1990), may be expected to increase the cytosolic Ca^{2+} concentration. As shown in Figure 9.4, a dose of 100 ng endothelin-2 (about 40 nmol/l) improved aortic output up to 10 minutes and then (40 minutes) worsened it compared to controls. The higher dose (200 ng) exerted a more consistent negative influence on aortic output recovery. These data are in agreement with the two-stage model of reperfusion stunning whereby agents thought to increase cytosolic Ca^{2+} first have an early positive inotropic effect followed by a later negative effect (Opie and Du Toit, 1992). Since the concentrations of endothelin required to produce these effects were about 3,500

times higher than those released upon reperfusion, released endothelin is unlikely to contribute to myocardial mechanical stunning.

There are at least three reasons why the present results may differ from those in humans. Although the isolated asanguineous Langendorff heart model is an accepted and widely used model, the crystalloid perfusion medium may have a different effect on endothelin release to whole blood. The coronary flow rate is also much higher in this model. Second, species differences cannot be ruled out and, third, endothelin internalized during ischaemia or reperfusion could not be measured.

SUMMARY AND CONCLUSIONS.

Our understanding of the mechanisms of reperfusion injury has improved significantly in recent years. The concept of reperfusion injury has rapidly evolved from being an issue of debate in the late 1980s to its present status where the involvement of either Ca^{2+} overload or oxygen derived free-radicals in reperfusion injury are both no longer questioned. The potential clinical relevance of reperfusion stunning in particular has now also been recognised (Bolli, 1992; Patel *et al.*, 1988; Kloner *et al.*, 1990) and will no doubt be the focus of numerous studies in the future.

The aim of this study was to investigate the effects of altered Ca^{2+} fluxes on the severity of reperfusion injury and the possible entry pathways contributing to the proposed Ca^{2+} overload.

Sarcolemmal Ca^{2+} entry pathways. Data (past and present) from our laboratory suggest that reversible reperfusion injury (arrhythmias and stunning) is modulated by interventions and compounds altering Ca^{2+} fluxes across both the sarcolemma and sarcoplasmic reticulum. In this study, pharmacologic (nisoldipine) and inorganic Ca^{2+} channel blockers (Mg^{2+} and Mn^{2+}) acting on the voltage dependent L-type channel, given at the onset of reperfusion, decreased the severity of reperfusion injury. By using a Ca^{2+} agonist (Bay K 8644), inotropes (isoproterenol or forskolin) or high Ca^{2+} concentrations during early reperfusion, subsequent stunning was exacerbated. These data suggest that interventions increasing early reperfusion Ca^{2+} fluxes through the L-type Ca^{2+} channel exacerbate stunning and favor the hypothesis that early intracellular Ca^{2+} overload may cause subsequent contractile dysfunction. These data also suggest that

inotropic interventions should be restricted to "late" reperfusion when the heart is less prone to Ca^{2+} induced reperfusion injury.

This study also provided conclusive evidence for a role for the Na^+/H^+ exchanger in the present model of reperfusion injury. The data presented in this thesis demonstrates that the novel and evidently very specific Na^+/H^+ exchange inhibitor HOE 694, when given before ischaemia, or at the *onset* of reperfusion, protects against reperfusion stunning and arrhythmias. The data also indicate that this compound is a more potent inhibitor of Na^+/H^+ exchange than amiloride at similar concentrations. Because Na^+/H^+ and Na^+/Ca^{2+} exchange are thought to be linked, our results again argue for a role of transient cytosolic Ca^{2+} overload in the genesis of these aspects of reperfusion injury. These data may be of great clinical relevance where myocardial reperfusion in the presence of this type of inhibitor could potentially attenuate these pathophysiological consequences of reperfusion. Na^+/H^+ exchange inhibition appears to be an effective means of attenuating reperfusion stunning and arrhythmias without jeopardising mechanical function of the heart. Although its clinical application has not been investigated, these inhibitors may offer a means of reducing reperfusion injury without the undesired inotropic effects still plaguing therapies based on Ca^{2+} channel blockers.

Intracellular Ca^{2+} fluxes and reperfusion injury. Inhibition of intracellular Ca^{2+} fluxes by compounds blocking Ca^{2+} release from the sarcoplasmic reticulum (ryanodine), or inhibiting sarcoplasmic reticulum Ca^{2+} ATP-ase pump activity (thapsigargin and cyclopiazonic acid) reduced reperfusion stunning and the incidence of ischaemic and reperfusion ventricular arrhythmias. Although the findings with the sarcoplasmic

reticulum Ca^{2+} ATP-ase pump inhibitors have shown promise, they are at present inconclusive as the possibility that these compounds may act on other sites in the myocyte cannot be excluded. Until the myocardial site of action of these compounds is better characterised, the exact mechanism by which these compounds protected against reperfusion injury remains inconclusive. Pretreatment of the isolated rat heart with these compounds decreased the severity of reperfusion stunning and/or ventricular arrhythmias in the rat heart, possibly by, i) inhibiting intracellular Ca^{2+} oscillations and/or, ii) by depleting the sarcoplasmic reticulum of Ca^{2+}.

Endothelin and reperfusion injury. Endothelin, thought by some to play a role in reperfusion stunning, is released in very small amounts in the rat heart during low-flow ischaemia. Although endothelin is released in fourfold higher concentrations during reperfusion, these small amounts are unlikely to cause or even contribute to mechanical stunning. A possible local effect of endothelin not released from the myocyte cannot however be excluded. Endothelin is a potent vasoconstrictor and may play a role in the development and/or aggravation of reperfusion vascular damage.

Reservation to the study. Although these studies were performed using pharmacological compounds to modulate Ca^{2+} fluxes, no cytosolic Ca^{2+} measurements were performed. In order to have conclusive evidence that increased cytosolic Ca^{2+} fluxes were associated with elevated cytosolic Ca^{2+} levels on reperfusion, I would have to perform cytosolic Ca^{2+} measurements by one of the non-invasive measuring techniques (bioluminescence, fluorescence or nuclear magnetic resonance techniques). These measurements fell beyond the scope of this study and should ideally be performed in the future.

CONCLUSIONS. This study therefore provides evidence that transsarcolemmal and intracellular Ca^{2+} fluxes during reperfusion play an important role in the genesis of reperfusion stunning and arrhythmias. These transsarcolemmal fluxes are mediated via Ca^{2+} channels, and/or the Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchangers. Furthermore, intracellular Ca^{2+} flux through the sarcoplasmic reticulum 1) ryanodine channel, and/or 2) the Ca^{2+} uptake pump, also appears to contribute to abnormalities of intracellular Ca^{2+} homeostasis and early reperfusion arrhythmias and stunning. By using pharmacologic compounds or interventions to modulate myocardial Ca^{2+} fluxes at the onset of reperfusion, the severity of reperfusion injury may be significantly reduced. These findings may have clinical relevance.

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